PLANT PHYSIOLOGY

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PLANT PHYSIOLOGY

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ADDRESS TO THE AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS[1]

CHARLES A. SHULL

42 Oakwood, Asheville, North Carolina

It was most gracious of you, Mr. President, to invite me to attend this meeting; and it is a very great pleasure to me, a rare privilege, to be present with you all on this happy occasion while we are celebrating together the 30th anniversary of the founding of this Society. I have often been asked to write a brief history of the early days of the American Society of Plant Physiologists, but have always declined because I could not do so without being put in the position of appraising many of my own activities; of detailing events that should be left to an impartial historian who could examine the facts objectively, and not overemphasize the part I played in those early days.

I would like to make it perfectly clear to all of you now, that it was not my privilege to have anything to do with the actual founding of the Society, even if I did serve as its first president. During the autumn of 1923, I was enjoying a very busy and time-consuming vacation from the University of Chicago. Specifically, I had been offered and had accepted a temporary post as assistant to the Secretary of the American Association for Advancement of Science (AAAS). In this position, I was commuting between Baltimore and Washington, D. C., three days a week, and continuing my soil-science research during the remainder of the week with some of the Livingston apparatus. In the Washington office of the AAAS during the four months preceding the Cincinnati meeting in December, 1923, not only was it my duty to handle a great deal of the Association's correspondence, but also to undertake the task of initiation of an exhibition of scientific apparatus, the first of its kind in the Association's history. This new venture required a great deal of time and planning. It proved so successful that it at once became a permanent part of the Association's program.

There was obviously no time to take much interest in what was going on, and what was rumored to be taking place, in plant physiological circles. On a few occasions, Dr. Livingston and I indulged in the pleasant pastime of sitting on opposite sides of a laboratory table, our feet appropriately propped up on the table, and exchanged views and speculated on what might be in prospect. But, as far as either of us could go, was to agree that, if there was to be a Society of Plant Physiologists, we ought to belong to it because of our respective positions in that field. Dr. Livingston was then the leader of the Johns Hopkins Laboratory of Plant Physiology, and I was recently appointed head of the Division of Plant Physiology at the University of Chicago. We felt that, under the circumstances, we ought to become members of any organized group that might arise to serve the interests of our particular field of Botany. It was not, however, until several months after the Cincinnati meeting that I joined the Society. Private personal records show that I paid my dues in the new organization on March 22, 1924. I was just an ordinary member, and I have always presumed that Dr. Livingston's connection was made in the same way. But neither of us, so far as I know, had anything to do with the formation of the Society of Plant Physiologists.

The germ of the Society had been lying like a dormant seed in the matrix of American botany through the upper teen-ages of the twentieth century. Like Ohga's Manchurian Lotus seeds lying in the ancient peat beds, it needed some exacerbation to change the conditions from dormancy to active germination. When germination actually started, I do not know. It began presumably in the frustration of plant physiologists at their own impotence. I had become aware of this feeling, for I was asked many, many times by thoughtful and worried colleagues, why plant physiologists never had a dinner at the annual conclave of botanists. I was too honest to suppress my real convictions, and always answered: "Societies have dinners; sections of societies do not!" The thoughtful, long faces which greeted this terse summary of the situation revealed that there were depths of thought, and brooding, in the hearts of plant physiologists. Moreover, there was the constant, more or less pompous parading of plant pathologists, and after 1915, of ecologists, with their annual dinners, president's addresses, and active support of their own objectives which struck deeply at the heart of the problem. It was not that the plant physiologists wanted to parade pompously in competition with anybody . . . perish the thought! But all of us felt that our field lay at the heart of botany; and we wanted to give our support, especially our financial support, to our own field.

Thus, while the roots of an organization were forming, the germination of the seed was peculiar, in that there was no sign as yet, of an emerging epicotyl. It was rumored that such visible germination might take place at the Chicago meeting in 1920. Few

[1] Presented at the Annual Meeting, September 6, 1954, Gainesville, Florida.

people know, perhaps, that germination was again delayed, presumably by quarantine regulations which kept the supposed leader of the plant physiologists at home, where some childhood diseases had erupted. At any rate, nothing happened. So it was not until the autumn of 1923 that definite growth of an epicotyl began. In the spring of 1924, if my memory is correct, two elections were held. It has always kept me from anything but a modest appraisal of my own importance to know that I was not the first choice of plant physiologists as first president of the Society. It was well toward the summer of 1924 that I was appraised of the fact that I had been elected president of the American Society of Plant Physiologists. This called for a deal of soul-searching, and non-hasty examination and appraisal of the possibilities. I had not sought the office, and at the time did not even know what had been transpiring. It was a sudden situation that confronted me, and the longer I pondered it the more certain I became that I had either to accept the responsibility if I were to remain a member of the new society, or I must resign my membership and state the reasons that led me to run out on my group.

During this difficult period there was much conflicting advice, and threadbare argument; but the memories kept coming back . . . the questions about the seeming neglect of physiology; and the longfaced questioners flashed across the scenes of memory. There was also the constant fear that there might not be enough interest to support a journal, even if we attempted to start one. It was probably a favorable circumstance that I was inexperienced, too young to recognize *impossibility* even when it stared us directly in the face. Somehow we could not realize the serious financial stumbling-blocks that would lie in our path. After several weeks of this soul-searching I came to the firm conclusion that the plant physiologists of my own age or younger, would never be satisfied until we had either succeeded, or had gone through a financial wringer for our ideals.

So the die was cast. The long struggle that followed concerned primarily the survival of the Society. For the problem of survival did fall upon my shoulders; and during a period of about 12 years it was the ever-present and dominant shadow that followed wherever I went. Though I was in no way involved in the Society's origin, I think that perhaps I did have something to do with the survival and early growth of this organization. Many of the things that were done in the early years were designed to keep up or increase the morale while time went by . . . time necessary for support to accrue. In many of our plans, Mrs. Shull shared in the decisions; and I want to record her early friendship for this group. We were contemplating the publication of a new journal when there was not one cent of money, nor any financial credit to support such a publication. To meet this situation, without mentioning names, we pledged three percent income on a $20,000 investment, or $600 per year for at least three years, to bolster the treasury. Payments on this pledge began in July, 1925, six months before the publication of PLANT PHYSIOLOGY was authorized by the Society. The payments were made quarterly, $150 per quarter, for five consecutive quarters, from July 1925 to July 1926, inclusive. I should not have to say that without Mrs. Shull's consent and earnest wish, this could not have been done.

A second step was taken after my term of office was ended. For a long time I had been interested in *financing for growth*. The idea itself was born during my early years as a young professor of biology, following in the footsteps of Rafinesque at the old Kentucky University, which had been immortalized in the novels of James Lane Allen and John Fox, Jr. The endowment of that oldest of educational institutions west of the Allegheny Mountains was inadequate; and I was figuring, one day, why it would not have been wiser to have put the $250,000 endowment to work creating endowment each year, than to have used the money directly for running expenses. That was it! *Endowed endowment!* Continual growth brought about internally by the proper use of endowment money. That idea lay dormant in my memory for nearly 20 years, until 1925 came along. It was during the summer of 1925, just after my term of office was closed that this problem of *endowed endowment* came to the surface, again. How could the Society provide money for continual growth? Even a hundred dollars a year would in a century provide a backlog of $10,000 which was far more than most Societies could boast. This idea became coupled in some way with the need for definite honors to be conferred upon those who over the years had given conspicuous service to plant physiology. Gradually the idea of the Charles Reid Barnes Life Memberships was evolved, and by the time of the 1925 meeting at Kansas City, Mrs. Shull and I had it clearly enough in mind that it could be presented in definite and clear form for Society acceptance or rejection. Nothing was said about the *financing for growth* or *endowed endowment*, but a simple offer of support of the Charles Reid Barnes memberships for a few years until the background support of the awards could be marshalled. The Society approved the idea unanimously, and each year for five years Mrs. Shull and I sent $100 to the treasurer in the fall of the year, for these awards. The morale factor in such awards was very high. In 1930 the break came for which we had been waiting. The secretary-treasurer came to the meetings with $1400 of unused money. The executive committee, including the secretary-treasurer, was finally persuaded to utilize that $1400, to start a Charles Reid Barnes Life Membership Endowment. In the course of the next four years, this endowment was increased to its present adequate support, thanks to the efforts of Dr. Livingston, who was elected president in 1934. It was for him a sort of tenth anniversary celebration. So came into being a small endowment, whose earnings, after supporting life memberships for a period of years, finally becomes

general endowment, thus providing the *endowed endowment* feature of our finance pattern. On an average, over the long years, the general endowment will grow at the rate of $100 per year, with no more thought on our part. Fortunately, also, it is not allowable to change such endowments, once they have become trust funds. So you have an *endowed endowment* as long as there is an American Society of Plant Physiologists.

The Kansas City meeting authorized the publication of PLANT PHYSIOLOGY as our research journal. The first number came out about three months later; and with the previous several quarterly payments on the pledged support, the treasurer was able to pay cash for the first number of PLANT PHYSIOLOGY. The effect of that first number was little short of phenomenal. Money poured in from new members, from library subscriptions, and from foreign countries in a steady flood. By July of 1926, when we made the last $150 payment to the secretary-treasurer, we knew that no more such payments would be required, especially in the immediate future. We were released from further payments for support of PLANT PHYSIOLOGY.

In 1927, contrary to my wishes, the Society once more elected me president of the group. The Constitution of the Society did not forbid it at the time; but we hastened to remedy that defect in the constitution. The annual meeting was to be held in Nashville, and as fourth president, I obtained permission to seek a Stephen Hales Endowment in honor of the 250th anniversary of Hales's birth, and the 200th anniversary of his great book, "Vegetable Staticks." As a result of personal solicitation, some 80 gifts were sent in totaling more than $900. In connection with his gifts to this fund, Dr. Livingston provided money for a first award in addition to his gifts to the permanent fund. It was intended that the first award would be made at New York in 1928, but the machinery could not be made to work in time, and the first award was made in 1929 to Dr. Dennis R. Hoagland for his excellent contributions to plant nutrition. A second award was made at Cleveland in 1930 to Dr. W. W. Garner for his development of the concept of photoperiodism in plants. So began the awards in even years for brilliant research, or for conspicuous service. Each step of the way was weighed in the balance of morale and survival.

During the decade of the 1930's this struggle went on, for there were still unused ways in which a part of our income could be impounded for permanent endowment. It was not easy to obtain consent for some of the changes; others came easily. Dr. Livingston's administration in 1934–35 was one of large changes. He redistributed the Society's assets in such a way as to make all of our funds adequate for the purposes to which they had been assigned. Bonds were reallocated to specific uses; and the constitution was rewritten to include PLANT PHYSIOLOGY within the structure of the Society. For, in our fears for its future, the journal had been established outside the Society, so that, in case it failed, it would not pull the Society down with it. Now it was boldly incorporated into the Society's Constitution. Some of you have wondered why we adhered to a fiscal year like that of the Federal Government. In the first years we had to know what the income would be; and how much expansion might be possible during the next year. It is the easiest thing in the world to destroy a society by unwisdom. With a year starting in July, we had our library subscriptions and membership funds nearly all in hand by the first of the year. As editor, I knew from the start of each year just how liberal we could be in use of our space. If your collections now do not begin until January, your editor will never know in time to trim his sails, if a hurricane strikes.

This is a brief and imperfect account of the struggle for survival which began with my first presidency, and continued all through my long connection as chairman of the Finance Committee, which began with the administration of Dr. Ezra Kraus, in 1928. I ought to mention with the highest praise, the faithfulness of the editorial assistants provided me after about ten years of single-handed editing of PLANT PHYSIOLOGY. These included Hazel Schmoll, Florence Brown, and Dr. J. Fisher Stanfield and Mrs. Stanfield after they came to Chicago. During the last most difficult years, Dr. Stanfield edited a large part of the manuscripts to relieve the pressure upon me as I was approaching the age of retirement. His present position as Executive Secretary-Treasurer began in 1944, as part of the reorganization made necessary by my removal from the Chicago area. Mrs. Stanfield has been a wonderful aid to him, I am sure.

There were two main reasons for giving up the editorship of the journal, PLANT PHYSIOLOGY, which by now was nationally and internationally famous. The library equipment in our new home town, while very unusual for a community of its size, was by no means adequate for the work that I felt had to be done on literature lists and other features of the editorial processes which involved library search. Secondly, I felt very strongly that the changes then sought should take place, not at my death or incapacitation, but at a time when I could have the satisfaction of seeing it go ahead as if nothing had really happened. I think the Society has done magnificently during the last ten years in taking over the entire responsibility. I am sure that my still youthful spirit has been prolonged in that state by being relieved. And I am profoundly grateful for the freedom which has been mine during these ten years.

I want to congratulate the Society, especially, on its increasing total worth, and its growing endowment strength. If you were using them solely for your own purposes, they would be a great element of strength in your financial structure. Much of the success of this region of the Society's interests rests again with Dr. Stanfield, who had caught the vision and the gleam during our richly rewarding cooperation. It is my most earnest wish that the policies set up and

pursued for growing strength be not disturbed. You are carrying on an experiment with *endowed endowments*, and I hope it may remain the permanent policy of the American Society of Plant Physiologists, to let this experiment run for a century or two, just to see what it can do. Nothing would be more thrilling to me than to see it result in a continued spectacular success. I have always been proud and glad that it was the plant physiologists who were reaping the rewards of prosperity and success. Let us do nothing to break the accumulation of strength in the financing of our work. In the future, generations of plant physiologists yet unborn will rise up to call you blessed, if you maintain what you have.

As a result of all these factors, we now have Plant Physiology dinners, without too much pomp and circumstance; we have a magazine which holds the respect of the world; we have considerable endowment, but not enough to worry anyone. Protected and conserved, in time it should give you some security, but nothing to waste. Personally, I have felt that the long struggle was worth the effort; and none of you can look back over the years in retrospect, with greater satisfaction.

The American Society of Plant Physiologists is, however, a tool for the invigoration and promotion of our research programs. It is the latter, the researches of this group, that make the most important contribution to the public good. Our research must go forward in two great realms. One of these deals with the responses of the plant to all kinds of environmental factors: temperature, water, light, nutrition, antibiotics, radiations other than light, etc., which if not superficial, are at least more or less external factors. This stream of investigation is important to any program of controlled plant production. It has large value in such special regions as floriculture, horticulture, forestry, agronomy, and related subjects. Plant management requires the constant application of this type of research.

To my mind, however, a far more important contribution must be made to an understanding of the plant's life. Every phase of the building of the plant's body is still in need of continuous research. The entire interior of the plant is made up of complicated physical and chemical systems, integrated and cooperating to carry forward the processes of building, of life, of decay, etc. Enormous ranges of physiology are still shrouded in mystery. This internal physiology is of the utmost importance to the proper understanding of life. It will require all of the skill of the biophysicists and biochemists, new techniques, and long patient research to unravel the complications of the internal chemistry and physics of the plant. As we go more and more deeply into these problems, they will necessarily become more complex, more confusing and confused, more difficult to untangle.

Enzymes, hormones, all kinds of biotic substances are involved in the manifestations of life. The origin of each reactant within the plant must be known, and the series of reactions which stand between the origin of material and the end result of reacting substances must be known and understood in all of their complexities before we can have a true picture of that internal mechanism. It is only by such an integration that we can understand even the origin of the plant's morphology. If one could only live through several centuries of research, it would be a thrilling experience to follow the gradual elucidation of all of the physiological processes in relation to each other.

It is my unshakeable belief that the organized work of this group can, in the long run, contribute immeasurably toward this more complete understanding of the processes whereby each plant comes to be what it is. It is fascinating to contemplate the slow but sure progress that is being made, and I rest in the complete assurance that, with the sound beginnings which have been made, and with the friendly fellowship which now pervades among all plant physiologists, the future will be ever more fruitful and satisfying to each and every one of you. It is my privilege to stand upon the shore, soon to shove off on new adventures, to wave my hands in fond greetings to all of you, and to wish you all the success which I am sure you will have richly deserved. Let me close with those famous words which the poet Catullus spoke at his brother's tomb: "Ave atque vale!" Hail, and farewell!

THE CALCIUM AND POTASSIUM NUTRITION OF COTTON AS INFLUENCED BY SODIUM[1,2]

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Under certain conditions sodium has been shown to exert a beneficial effect on the growth and yield of cotton (1, 3, 4, 10, 12). Most investigators emphasize the relationship between sodium and potassium, stating that sodium is most effective in increasing yields of cotton on soils deficient in potassium. Data from Cooper and Garman's paper (3) indicate that rather uniform gains due to sodium of 182 to 215 pounds of seed cotton resulted from the addition of sodium nitrate to their plots. Marshall and Sturgis

[1] Received June 3, 1954.

[2] Published with the approval of the Director of the Texas Agricultural Experiment Station as Technical Paper No. 1983.

(10) reported $NaNO_3$ gave significantly higher yields than did NH_4NO_3 in seven experiments when used in complete fertilizers carrying high amounts of potash. Lancaster et al (9) noted that the largest increase in yield of cotton attributed to sodium was obtained on soils deficient in potassium; however, in two instances, $NaNO_3$ was superior to NH_4NO_3-lime even with adequate potassium. Experiments conducted by Wallace et al (14) with alfalfa tend to support the idea that sodium is effective in increasing yields only under conditions of low potassium fertility. The latter investigators conclude that since most of the sodium was in the stems of the alfalfa plant, the function of sodium must be a simple replacement of potassium rather than a physiological function of sodium. The cation content of the above ground portion of the cotton plant has been studied by Cooper, Garman and others (3, 4, 7). It is reported that sodium has little or no effect on the absorption of potassium but does depress the uptake of calcium. Potassium, on the other hand, has a very marked effect on the absorption of sodium and calcium. These investigators report that optimum yields of cotton were obtained when the Ca/K + Na ratio in the plant is small. Cooper et al (4) suggest that the ratio would be lower for maximum yield than the 1.41 reported in their paper, and may be near unity. In an earlier paper Cooper and Garman (3) suggest an inverse relation between the Ca/K + Na ratio and yield. Wadleigh (13), using very dilute solutions, noted a direct relationship between green weight of cotton plants and proportioned parts of potassium in his nutrient solution. Medium concentrations of calcium and magnesium gave optimum results for those elements.

The present investigation was undertaken to study: (1) the absorption and distribution of cations in the cotton plant, (2) the influence of sodium on the growth and yield of cotton in the presence and absence of adequate amounts of potassium and calcium and, (3) to determine the relationship between the Ca/K + Na ratio and yield.

Experimental Methods and Results

Sand Culture Experiment: Cotton seeds, variety Stoneville 2B, were planted in forty 3-gallon glazed jars containing washed creek sand. Ten days after emergence the seedlings were thinned to three per jar and nutrient treatments were started. A modified Hoagland's solution was used as the control and four variations of this solution were employed. Each of the four nutrient treatments received solutions containing the following salts.

Salt	Millimolar concentration
$Ca(NO_3) \cdot 4\,H_2O$	5
$Mg\,SO_4 \cdot 7\,H_2O$	2
$NH_4H_2PO_4$	1
Trace elements B, Mn, Zn, Cu, Mo	

In addition KCl and NaCl were supplied to the four treatments as follows:

Salt	Millimolar concentration
Treatment I (high K, high Na)	
KCl	5
NaCl	10
Treatment II (low K, high Na)	
KCl	0.5
NaCl	10
Treatment III (high K, low Na)	
KCl	5
NaCl	0
Treatment IV (low K, low Na)	
KCl	0.5
NaCl	0

Finely ground commercial magnetite was added to the sand as a source of iron (5). Sufficient quantities of the other minor elements were contained in the salts and sand employed.

Each jar was provided with a Pyrex glass side arm which was marked at a level corresponding to 10 cm below the surface of the sand. The pots were drained daily and then filled with sufficient nutrient solution to bring the liquid level to the mark on the side arm.

Fifty days after emergence, ten plants were harvested from each of the four treatments and determinations of total Na, K, and Ca were made, using a Beckman flame photometer. At this time no significant differences in growth were noted between treatments.

Distribution of Cations in 50-day-old Cotton Plants: In this experiment sodium tended to accumulate in the roots of the cotton plant (table I). In general about one fifth as much Na was contained in the stems and old leaves as in the root, while the

Table I

The Distribution of Na, K and Ca in 50-day-old Cotton Plants as Influenced by Various Substrate Levels of Na and K *

Plant fraction	Na	K	Ca	Total Na + K + Ca
I (Control—High Na, High K)				
Root	49.32	41.07	9.73	100.12
Stem	9.06	32.07	28.81	70.94
Old leaves	9.49	46.89	113.65	170.03
Young leaves ..	4.56	44.79	66.82	116.17
II (High Na, Low K)				
Root	71.89	10.68	11.42	93.97
Stem	16.90	4.97	46.21	68.08
Old leaves	16.94	8.24	145.20	170.38
Young leaves ..	12.59	19.86	90.18	122.63
III (Low Na, High K)				
Root	32.72	47.92	9.17	89.81
Stem	7.22	35.26	29.28	71.71
Old leaves	5.96	46.60	119.53	172.09
Young leaves ..	3.73	44.56	66.53	114.82
IV (Low Na, Low K)				
Root	48.95	10.41	9.98	69.34
Stem	9.53	5.33	38.45	53.31
Old leaves	8.94	6.21	143.4	158.55
Young leaves ..	5.96	19.17	84.43	109.56

* Results expressed as meq/100 gm dry wt.

young leaves contained the least sodium. Uniform concentrations of K were found in the root, old leaves and young leaves, whereas the stems contained about three fourths as much. The roots contained the lowest concentration of Ca. The Ca level increased 3-fold in going from the roots to the stem. A very striking accumulation of Ca was noted in the old leaves, being approximately a twelve-fold increase over the roots. The young leaves contained a level of Ca about half that of the old leaves. These results agree closely with those of Mason and Phillis (11) for potassium and calcium content of cotton leaves.

When only Na was omitted from the nutrient solution, a 25 % reduction of Na was noted in all tissues, however, this loss in Na was counterbalanced by slight increases in K or Ca and the meq sum of Na + K + Ca remained the same as in the controls. When K was the limiting element, marked reductions in K content were observed, while Na and Ca increased in all plant fractions. The largest increase in Na was noted in the young leaves which accumulated 2.78 times as much Na as the controls. The stems of the low-K plants showed the sharpest rise in Ca content. The loss in K was offset by increased Na and Ca, and here, as in the low-Na plants, the total Na + K + Ca value remained the same as in the controls.

In treatment IV (low Na, low K) marked decreases in Na and K were accompanied by an increase in Ca. It is noted, however, that the Ca content of the various plant fractions in treatment IV did not reach the level obtained in treatment II (high Na, low K). Of the treatments employed only number IV (low Na, low K) had an influence on the sum of cations (Na + K + Ca). In this series a notable reduction in cation sum was obtained.

THE CATION NUTRITION OF COTTON AS INDICATED BY HYDROPONIC EXPERIMENTS: On December 6, 1952 Stoneville 2B cotton seedlings were transplanted from sand flats to 5 gallon jars containing nutrient solution. Three seedlings were placed in each of 24 jars.

For the first 45 days only two treatments were applied. The control series, 20 jars, received a solution containing millimolar concentrations of salts as follows: 5 NH_4NO_3, 2 $MgSO_4 \cdot 7\ H_2O$, 3 $CaCl_2$, 2 KCl, 1 $NH_4H_2PO_4$. Minor elements were added to insure a sufficient supply. The control + Na series, 4 jars, received the same solution plus 10 millimoles NaCl. Each jar was supplied with a porous clay aerator attached to the central air line and a pressure of 5 lbs/in^2 was maintained during the experiment. Forty-five days after transplanting four representative plants were harvested from each treatment with only one plant taken from any given jar. These plants were fractionated into roots, stems and leaves and determinations of Ca, Na, and K were made. At this time four new treatments were started. The control series was divided into five groups of 4 jars each. These treatments were designated as control, − K, − K + Na, − Ca, and − Ca + Na. The control series continued to receive the same nutrient solution as previously mentioned. In both the − K and − K + Na treatments, KCl was omitted from the solution whereas $CaCl_2$ was the only salt missing in the − Ca and − Ca + Na solutions. Ten millimoles of NaCl were added to the − K + Na and − Ca + Na treatments. The control + Na treatment was continued as previously outlined. All jars were drained, washed and filled with freshly prepared solutions each week. The pH of each solution was checked daily and control was maintained between limits of 5.0 to 6.3 by the addition of dilute HNO_3 or NH_4OH. The experimental design was a randomized block with 4 replications on the greenhouse bench.

When the plants were 90 days of age a second

TABLE II

DRY WEIGHT OF 45- AND 90-DAY-OLD COTTON PLANTS GROWN ON WATER CULTURES AS INFLUENCED BY VARIOUS CATION TREATMENTS. RESULTS IN GM

PLANT FRACTION	45-DAY HARVEST		90-DAY HARVEST					
	CONT.	CONT. + NA	CONT.	CONT. + NA	ZERO K	ZERO K + NA	ZERO CA	ZERO CA + NA
Root	4.65	3.94	8.96	8.42	9.91	9.85	5.58	7.61
Stem	3.22	3.13						
lower			5.36	5.63	7.10	5.96	3.97	4.54
upper			0.89	0.97	1.97	0.64	0.51	0.82
Branches			3.53	2.70	4.30	2.62	2.06	2.07
Leaves	7.12	7.43						
lower			4.73	4.31	5.50	6.32	2.29	5.85
upper			3.63	3.77	5.28	3.61	2.30	4.27
shed			....	....	....	....	5.56	....
Branch leaves								
lower			4.52	3.31	5.69	3.52	2.67	3.05
upper			6.01	4.14	6.60	5.90	3.42	4.30
Fruit			25.39	20.99 *	12.49 **	23.95	1.10 **	10.32 **
Total	14.99	14.50	63.02	54.24	58.84	62.38	29.84 **	43.38 *
Ratio dry wt bolls / stems + leaves			0.886	0.845	0.343	0.838	0.063	0.408

* Significant to .05 level.
** Significant to .01 level.

FIG. 1. The influence of additional sodium (10 mM) on cotton plants grown on solutions deficient in calcium.

harvest was made. Four representative plants from each treatment were fractionated and chemical determinations were made.

The experiment was repeated in the winter of 1953 to 1954. Since results of the two experiments were almost identical only those obtained in 1952 are presented.

Growth and general appearance of plants: For the first 45 days of the experiment all of the plants were grown on a complete nutrient solution, with or without Na. During this period no difference was noted between plants receiving Na and those grown in the control solution (table II).

On the 46th day the various treatments were started. Eight days after starting the various treatments it was observed that the – Ca plants were wilting easily and that their leaves were a pale green color while the – Ca + Na plants appeared normal. Photographs were taken of the – Ca and – Ca + Na plants (fig 1). At that time it was apparent that the – Ca plants would not survive, and $CaCl_2$ was added to both the – Ca and – Ca + Na series at a concentration 1/40 that of Hoagland's solution. At the 90-day harvest it was noted that the – Ca plants had a dry weight increase of about 15 gm when compared to the 45-day-old control plants, while a 29 gm increase was recorded for the – Ca + Na series in the same period. Table II shows that a large percentage of this increased dry weight due to sodium may be accounted for in the weight of fruit.

TABLE III

NA, K AND CA CONTENT OF 45-DAY-OLD COTTON PLANTS GROWN IN WATER CULTURES

TREATMENT	PLANT FRACTION	TOTAL MEQ			MEQ/100 GM		
		NA	K	CA	NA	K	CA
Control	leaves	0.51	3.29	3.17	7.14	46.14	44.53
	stem	0.27	1.16	0.53	8.52	35.87	16.44
	root	0.37	1.60	0.37	7.84	34.32	7.88
	Total	1.15	6.05	4.07			
Control + Na	leaves	1.50	3.51	3.11	20.15	47.26	41.88
	stem	0.67	1.38	0.53	22.35	44.17	16.95
	root	0.66	1.76	0.47	16.71	44.72	11.91
	Total	2.83	6.65	4.11			

TABLE IV

THE TOTAL MILLIEQUIVALENTS OF NA, K AND CA IN VARIOUS PLANT FRACTIONS OF 90-DAY-OLD COTTON AS INFLUENCED BY NUTRIENT SUPPLY

TREATMENT	CATION	PLANT FRACTION						MEQ K + CA + NA PER 100 GM
		ROOT	STEM + LEAVES		SHED LEAVES	FRUIT	TOTAL	
			I *	II **				
Control	Na	0.81	1.03	1.79	...	1.01	4.64	
	K	2.45	3.19	6.54	...	8.21	20.39	67.16
	Ca	0.96	4.42	8.71	...	3.20	17.29	
Control + Na	Na	1.36	2.46	3.99	...	1.75	9.56	
	K	2.33	2.72	5.20	...	6.92	17.17	73.12
	Ca	0.84	3.46	6.10	...	2.62	13.01	
– K	Na	1.61	1.66	3.34	...	0.78	7.39	
	K	1.66	1.52	2.91	...	3.21	9.30	60.01
	Ca	0.98	4.86	10.54	...	2.22	18.60	
– K + Na	Na	2.13	2.70	4.37	...	1.96	11.16	
	K	1.45	1.48	2.10	...	4.71	9.74	64.67
	Ca	0.92	5.17	7.72	...	2.67	16.48	
– Ca	Na	0.61	0.64	1.14	0.54	0.10	3.02	
	K	1.48	2.03	5.82	2.78	0.81	12.92	76.17
	Ca	0.37	1.12	2.13	3.02	0.16	6.80	
– Ca + Na	Na	1.40	2.62	3.52	...	1.04	8.58	
	K	1.94	3.54	6.01	...	3.96	15.45	69.44
	Ca	0.52	2.84	1.99	...	0.68	6.03	

* First growth 0–45 days.
** Second growth 46–90 days.

When the plants were 65 days of age both the – K and – K + Na plants showed symptoms typical of K deficiency. The symptoms were not confined to the old leaves but rather appeared on all leaves regardless of age or position on the plant. As the plants aged the symptoms of K deficiency grew more severe; however, no observable difference could be detected between the – K and – K + Na plants. The total growth of both series was nearly equal and no obvious difference was noted between these treatments and the control plants. On closer examination of table II a trend becomes apparent between the – K and – K + Na plants. The addition of Na resulted in a shift from vegetative growth to fruiting and increased yields were obtained.

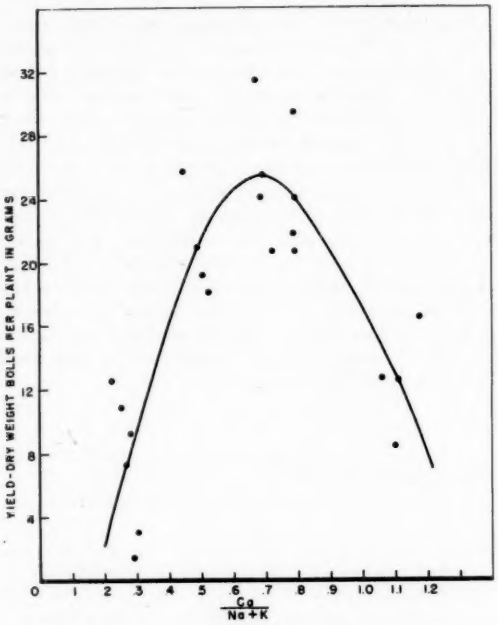

FIG. 2. The relation between cation ratio and yield of cotton.

Cation content of plants: Table III presents the results of analyses of the 45-day harvest. In general the distribution of K and Ca in the plants grown in water cultures agrees with the data previously presented for 50-day-old cotton grown in sand. A notable exception is seen in the response to Na when the two experiments are compared (table I and table III). The plants grown in water cultures did not accumulate large quantities of Na in the roots; also the water culture plants maintained higher concentrations of Na in the above-ground portions in comparison to the sand culture plants.

The results of the 90-day harvest are presented in table IV. When K was omitted from the nutrient solution, increased absorption of Ca and Na occurred (table IV). The addition of Na to the nutrient solutions decreased Ca absorption in all cases but had little effect on the uptake of K. When Ca was the deficient element the absorption of both Na and K decreased.

Cation ratio and yield: Cooper and Garman (3) have suggested that yields of cotton can be expressed as an inverse function of the Ca/Na + K ratio. Thus as this ratio becomes small yields of cotton increase. This seems to be true in the ranges studied by these investigators but it evidently is not a complete picture. As seen in figure 2 the relation between yield and cation ratio is parabolic, and in this experiment maximum yields were obtained with a ratio of approximately 0.7. Above or below this value noticeable declines in yield were obtained.

DISCUSSION AND CONCLUSIONS

Sodium played an important role in cation nutrition of cotton in experiments where plants were grown on complete nutrient solutions for 45 days and on solutions which were deficient in either Ca or K for a second 45-day period. Plants grown on the – K and – K + Na solutions accumulated about 3 meq of K during the second 45-day period indicating these solutions actually contained a small amount of available K. Within eight days after Ca was omitted from the nutrient solution the – Ca plants were wilting easily while the – Ca + Na plants appeared normal. At this time Ca was added to both the – Ca and – Ca + Na series at 1/40 the concentration contained in Hoagland's solution. As the experiment progressed it became apparent that Na was playing some role in substituting for Ca, since the – Ca + Na plants were in better condition and produced more fruit than the – Ca series. This function is independent of the oft mentioned role in replacement of K, since both solutions contained ample amounts of the latter element. Several possibilities seem apparent in evaluation of the role of Na in relation to Ca. First, Na had some influence on the absorption of Ca from solutions deficient in that element, or, secondly, a more efficient utilization and redistribution of absorbed Ca took place in the presence of additional Na. Table IV shows that Na had no influence on Ca absorption since the – Ca plants contained slightly more Ca than did the – Ca + Na plants (6.81 to 6.02 meq Ca/plant). Of the total Ca absorbed, the – Ca plants lost 3.02 meq per plant by leaf shedding, whereas in the presence of additional Na leaf shedding did not occur, and it may be that some of the Ca in the lower leaves became available for fruiting and other vital physiological processes. That this is true can be seen by comparing data in tables III and IV. The control plants in table III had a total Ca content of 4.07 meq at 45 days while 45 days later the same tissues in the – Ca series (roots, stem leaves 0–45 days and shed leaves) accounted for 4.51 meq Ca (table IV). When Na is considered, the same tissues in the – Ca + Na series (root, stem leaves 0–45 days) contained only 3.35, a net loss of about 0.7 meq Ca at the 90 day harvest. This quantity of Ca corresponds very closely

to that moved into the bolls (0.68 meq Ca) by those plants. Another possibility is concerned with the observed leaf shedding in the −Ca plants and the absence of shedding when Na was added. Shedding was apparently stimulated by the desiccation of leaf blade tissue and occurred in the absence of Ca; however, when Na was added the leaf blades remained turgid and shedding did not occur. This may suggest some indirect role of Na in hydration of tissues or water absorption.

In this experiment Na had no effect on the visual symptoms of K deficiency in the −K and −K+Na series. It is also noted that Na had no effect on total growth of plants in these solutions, nor was any difference noted between the two series in the absorption of K. The K absorbed in the first 45 days of this experiment (about 6 meq per plant), and the additional amount absorbed by the −K and −K+Na plants during the last 45-day period (3 meq per plant) was sufficient to permit vegetative growth to continue at a normal rate, but in the absence of additional Na fruiting activity was sharply limited. In contrast to Ca, K was very mobile in the plant and moved out of the old tissues readily. This movement of K was apparently independent of Na.

The role of Na in substitution for K and Ca is emphasized in the ratio of dry weight of bolls over dry weight of stems plus leaves, table II. This ratio, although not directly comparable to relative fruitfulness (the number of bolls per 100 gm fresh top weight defined by Eaton and Rigler (6)) serves to indicate trends in fruiting behavior. In the presence of adequate K and Ca, Na had no influence on the ratio of bolls to stems plus leaves, since the control and control + Na series had respective ratios of 0.89 and 0.85. When K supply was reduced the ratio dropped to 0.34; however, the −K+Na plants had a ratio of 0.84, nearly equal to the controls. The −Ca and −Ca+Na series showed a similar trend with ratios of 0.06 and 0.41 respectively.

The data presented in this paper seem to agree in part with conclusions drawn by Bear and Prince (2). They state that cations (Ca, K, Mg) have two functions in the plant; one specific and the other general which can be performed by all three ions interchangeably. Since Na is shown by the present data to substitute to some extent for K and for Ca, support is provided for the idea of a general function of cations. Further examination of the data suggests a common function of Na and K or of Na and Ca; however, as may be noted in the −Ca and −Ca+Na series, increased K concentrations in the plant failed to substitute for Ca deficiency in terms of yield. Bear and Prince noted no relation between yield of alfalfa and Ca/K ration. In cotton a parabolic relationship is established between yield and cation ration (Ca/K+Na). The apparent difference between the two observations may be explained by the fact that alfalfa yield was measured as total top growth while yield of cotton is in terms of reproductive activity.

Summary

The accumulation and distribution of Na, Ca and K in the cotton plant when grown in sand cultures was compared to similar plants grown in water solutions. A difference between results from the two experiments was found in the loci of Na accumulation. Less Na accumulated in the roots and more in the tops of plants grown in water.

When 45-day-old cotton plants were deprived of their Ca supply there was an apparent change in the ability of the plants to absorb water and within five days severe wilting was noted. The addition of Na to such solutions prevented wilting and the plants appeared normal in the absence of Ca.

Sodium was found to have a marked influence on the fruiting activity of 90-day-old plants grown on nutrient solutions deficient in either K or Ca during the last 45 days of their growth period. The role of Na in substitution for Ca may be a mobilization of stored Ca in the older tissues. The amount of Ca moved out of those tissues when Na was present was nearly equal to the amount of Ca accumulated in the bolls of those plants. There was no influence of Na on K movement, thus the role of Na in substitution for K in cotton can not be ascribed to the replacement of K in one organ of the plant thereby permitting its reutilization by other more active tissues.

A parabolic relationship was found to exist between cation ration (Ca/K+Na) and yield of cotton. A ratio of about 0.7 gave maximum yields, with noticeable declines on both sides of that number.

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THE PROPERTIES OF JANUS GREEN B AS A HILL OXIDANT[1,2]

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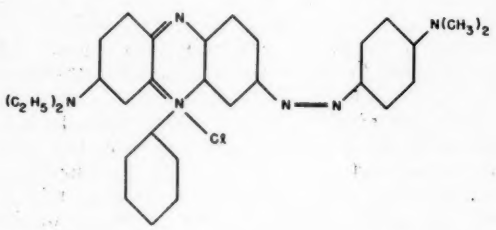
Beginning in 1937 in a series of papers (8, 9, 10, 11), Hill showed that in the presence of ferric ion, freshly isolated chloroplasts will, when illuminated, give a sustained production of oxygen equivalent to the ferric ion present. Later workers (16) added a considerable number of substances to the list of Hill oxidants. In the present paper Janus Green B is, for the first time, shown to act as a Hill oxidant.

In the presence of illuminated chloroplasts, it is changed from its blue to a red form. No change in color is observed if the dye is illuminated without chloroplasts, if the dye plus chloroplast mixture is unilluminated, or if the chloroplasts first are heated at about 50° C for a half-hour. This treatment does not cause the noticeable flocculation which heating at higher temperatures brings about, and a microscopic comparison of these heated chloroplasts and unheated ones revealed no obvious differences.

Janus Green B has certain peculiar properties as a Hill oxidant which set it apart from two other widely used Hill oxidants: quinone (5) and Hill's solution (12); certain of these properties suggest that Janus Green B is reduced by a different mechanism from quinone or Hill's solution.

Materials and Methods

Janus Green B: The Janus Green B used in the experiments to be described has, according to its manufacturer, the following structural formula.



The name Janus Green B has been used in the literature for a variety of very similar dyes, differing mostly in the positions and presence of methyl and ethyl groups.

The reduction of Janus Green B by illuminated chloroplasts apparently is the same as that which has been frequently observed to take place in the dark in living cells of both plant and animal material (2, 14, 18): the reductive splitting of Janus Green B into diethyl safranine and dimethyl-paraphenylenediamine. Since the Janus Green B is adsorbed almost completely by chloroplasts, whereas the red reduction product remains in solution, one can easily obtain a light absorption spectrum of the latter by centrifuging down the chloroplasts, along with any remaining Janus Green B which is bound to the chloroplasts, and observing the supernatant fluid. A spectrum of the reduction product obtained in this way with a Beckman DU spectrophotometer is shown in figure 1, where the two subsidiary peaks at 675 mμ and 445 mμ are due to some chloroplast material remaining in suspension. The character of the spectrum around 555 mμ is identical with that published for diethyl safranine (6). The spectrum of the Janus Green B used in these experiments, also shown in figure 1, is likewise identical with published data (6).

It is possible to reduce Janus Green B through a series of steps finally ending up with leuco diethyl safranine and dimethyl-paraphenylenediamine. The first step in the reduction of Janus Green B is reversi-

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Part of the work described here was done while the author was the recipient of the Conway MacMillan Fellowship of the University of Minnesota.

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ble and yields a leuco Janus Green B, whose structure has not been established (2, 13). Further reduction causes the rupture of the azo bond and liberation of diethyl safranine and dimethyl-paraphenylenediamine. This reductive step is usually regarded as irreversible (13, 15, 17). However, there are data in the literature which allow one to attach a more or less meaningful value to the "reduction potential" of this step. Rapkine et al (17) were able to obtain in acid, but not in alkaline solution, a characteristic titration curve with a point of inflection corresponding to the normal electrode potential of the reduction of Janus Green B to a red dye. By assuming an increase in normal electrode potential of 0.06 volts per pH unit, which applies to substances that in the process of reduction acquire hydrogen and whose acid dissociation constants are small compared to the hydrogen ion concentration of the solution (15), and extrapolating the data of Rapkin et al, one obtains for the normal electrode potential at pH 6.6 a value of about + .02 volts.

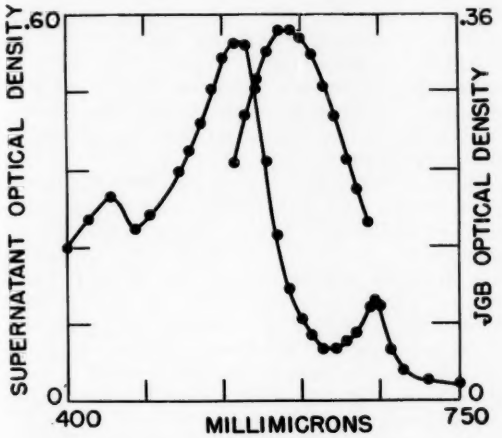
The value for the normal electrode potential of Janus Green B at pH 7 of 0.256 volts, which is sometimes quoted, refers to the reduction of diethyl safranine to leuco diethyl safranine. This high potential reductive step apparently did not occur in the experiments reported in this paper.

GENERAL EXPERIMENTAL DETAILS: Chloroplasts for the experiments were obtained either from Swiss chard grown in the Botany Department greenhouse or from fresh market spinach. To obtain a chloroplast suspension leaves were cut into pieces (after removal of the midrib in the case of Swiss chard) in a dimly lit cold room at 2° C and homogenized for 15 to 25 seconds in a Waring blendor with M/15 potassium phosphate buffer of pH 6.5 to 6.6 and containing 0.05 % KCl. The homogenate was strained through cheesecloth and centrifuged at 2000 × g for 12 minutes. The supernatant fluid was discarded and the sediment brought up to the desired concentration with a solution of the same composition as was used to blend the leaves. The suspension, thus obtained, was stored in dry ice until immediately before use, which treatment was presumed to hold the chloroplasts constant in activity over the storage period not exceeding about two weeks (7). This procedure was only varied in that sometimes the buffer solution also contained 0.5 M sucrose.

Chlorophyll was estimated by the method of Arnon (1).

In all experiments in which chloroplasts were illuminated in the process of measuring gas production, hydrogen, which had passed through a "Deoxo" catalytic deoxygenation cartridge, constituted the gas phase.

The Janus Green B was obtained from the Coleman and Bell Company, and they claimed the preparation to be only 54.1 % pure. It was used in these



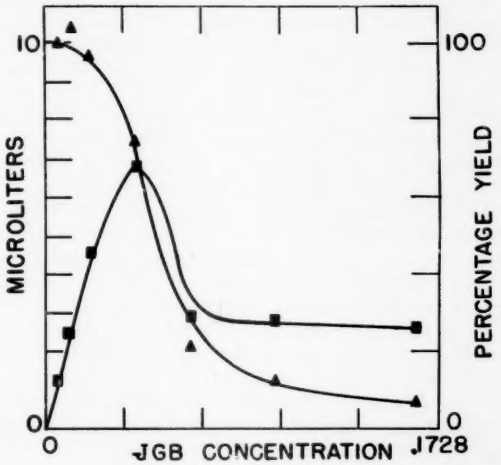


FIG. 1 (*left*). Absorption spectrum of the supernatant of a centrifuged chloroplast suspension after it has undergone a Hill reaction with Janus Green B compared to the absorption spectrum of an aqueous solution of Janus Green B. Abscissa is wavelength in mμ. Left hand ordinate is the optical density of the supernatant. Right hand ordinate is the optical density of the aqueous Janus Green B solution. The three-peaked curve refers to the supernatant and the single peaked curve to the aqueous Janus Green B solution.

FIG. 2 (*right*). Relationship between oxygen yield in the Janus Green B Hill reaction and Janus Green B concentration, using Swiss chard chloroplasts. Abscissa is initial concentration of Janus Green B in the reaction medium in gm/l. Left hand ordinate referring to squares is the oxygen yield in μl. Right hand ordinate referring to triangles is the percentage yield of oxygen based on a reaction stoichiometry where, for each oxygen molecule released, a Janus Green B molecule is split reductively. The chlorophyll concentration is 0.34 grams per liter. Temperature is 11.8° C.

experiments without further purification, and all calculations of dye concentration are based on dye constituting only 54.1 % of the dye preparation.

Results

The Oxygen Yield in the Janus Green B Hill Reaction: Only small amounts of oxygen could be obtained using Janus Green B as a Hill oxidant. The relationship between stoichiometry of the reaction and dye concentration, for a given chloroplast suspension density, is shown in figure 2. These data were obtained using Warburg manometers with vessel constants ranging from 0.59 to 0.65 μl O_2/mm of Brodie, and some of the smaller yields, therefore, actually came close to the limit of sensitivity of the method employed for measurement. In order to exclude, as far as possible, complications from heating effects within the vessel when the light is turned on, an acid ferrous sulphate filter was interposed between the light source and the constant temperature bath. Furthermore, two precautions were taken to account for any such heating effects which might still occur: 1) blanks, containing heat "killed," but not flocculated, chloroplasts plus dye, were run, and 2) after the completion of a light period, careful readings were taken in the immediately subsequent dark period to see if any rapid apparent gas uptake appears. Usually no heat correction was necessary, and the largest one ever used was two millimeters of Brodie which is equivalent to about 1.2 μl of gas.

The falling-off of oxygen yield with increased dye concentration was not due simply to shading of the chloroplasts by the dye resulting in rates so low that the reaction never had sufficient time to run its course, since gas evolution occurred only in the first part of the light period and was followed by a long interval in the light with no observable gas exchange.

In trying to explain the peculiar curve of figure 2, one is faced with at least these obvious alternatives:

1. Something happening in the light destroys the ability of chloroplasts to perform the Janus Green B Hill reaction, and, the more concentrated the Janus Green B, the sooner is the reaction "killed" relative to 100 % stoichiometry.

2. In photosynthesis and the Hill reaction, oxidizing as well as reducing power is produced in the light; the latter causes the reduction of carbon dioxide or Hill oxidant, while the former expresses itself ordinarily as a production of oxygen. The curve of figure 2 might be explained by supposing that the final yield of oxygen observed is the result of a balance between light-dependent reducing reactions, on the one hand, and light-dependent oxidizing reactions, which oxidize reduced Janus Green B while using up the oxidizing power that ordinarily appears as oxygen, on the other. (This mechanism, in the simple form expressed here, requires that the "irreversible" reduction step to produce diethyl safranine be reversible, which is probably unlikely. Further elaboration of the mechanism would allow one to circumvent this difficulty, but it is felt that the extent of the data available does not warrant elaborate theorizing.)

In order to distinguish these two possibilities, "double dose" experiments were run, wherein, after a first reaction with Janus Green B had run its course, a second dose of dye was added and its reaction observed. If, after a first dose of a concentration putting the reaction on the part of the curve of figure 2 to the left of the maximum, a second similar dose was added, then the oxygen yield of the second dose closely aproached that of the first. However, a second dose of a concentration putting the reaction on the curve to the right of the maximum, following a first dose of the same concentration, resulted in a several-fold smaller stoichiometry for the second dose, compared with the first. The fact that some oxygen is evolved from the second dose, even on the falling part of the curve, eliminates the first of the two alternatives mentioned above, since, if the ability of illuminated chloroplasts to cause oxygen evolution with Janus Green B had been destroyed by the first dose, a second dose should be ineffectual in causing further oxygen evolution.

The Janus Green B Hill Reaction Compared with Other Hill Reactions: Since the gas yields with Janus Green B, relative to the volume of material needed to attain these yields, were so small, it was necessary, in order to study the Janus Green B reaction in more detail, to resort to a more sensitive method for measuring gas exchange than Warburg manometry provides. The recording mass spectrometer described by Brown et al (4) was used to make the measurements of oxygen production described in the remainder of this paper.

The experimental material was held in a Warburg type vessel shaken in a constant temperature bath, with the leak mounted on the male joint just above the vessel (3), and to avoid complications from heating effects, measures, like those previously described, were also taken here. All reports of oxygen partial pressure in the remainder of this paper are in arbitrary units which happen to correspond to millimeters of peak height on the Brown recorder chart with the spectrometer data taken at the highest sensitivity.

The experiments with the mass spectrometer indicate that:

a. For a given light intensity at the chloroplast and with other factors similar, Hill's solution can cause a far faster oxygen evolution from illuminated chloroplasts than can Janus Green B.

b. The inhibition of the rate of oxygen evolution from illuminated chloroplasts, which a first dose of Janus Green B exercises over a subsequent dose of Janus Green B, is, percentagewise, far greater than the inhibition over a subsequent dose of Hill's solution or quinone.

The first result (a) arises from an experiment in which three successive 0.5 ml doses of a Hill oxidant were added, each before a separate light period, to 5 ml of a Swiss chard chloroplast suspension containing 0.75 mg of chlorophyll and held at 20.6° C. The

first dose of Janus Green B, containing 0.35 mg of dye, caused an initial rate of oxygen production of 14.3 mm per minute The second dose, containing the same amount of dye, caused an initial rate of oxygen production of 2.8 mm per minute. However, a third dose of Hill's solution caused a rate of oxygen evolution more than six times the initial rate for the first dose. Figure 3 illustrates some of the data upon which these quantitative statements are based.

A rigorously meaningful quantitative comparison of the rates for the first and the third dose is not possible since no estimate was obtained of where on the light saturation curves the various reactions lie, and, due to the occurrence of preceding Hill reactions, subsequent ones must run under somewhat different conditions from the first ones. Nevertheless, since all differences in conditions between the first and third

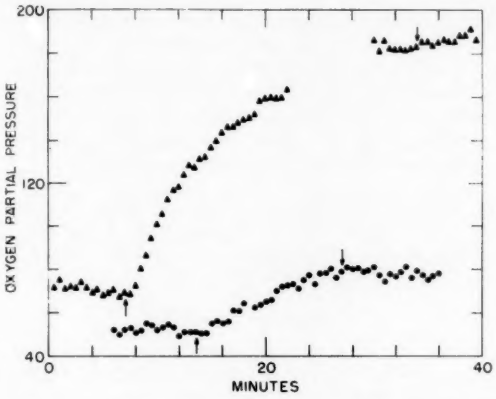


FIG. 3. Oxygen liberation from first and second dose of Janus Green B in the presence of illuminated chloroplasts. The abscissa is time in min. The ordinate is partial pressure of oxygen in arbitrary units. The triangles are for the first dose of Janus Green B and the closed circles are for the second dose. The up-pointing arrows indicate when the light was turned on and down-pointing arrows indicate when it was turned off.

dose reaction, like shading of chloroplasts by dye, inhibitory effects of dye or reaction products, inhibitory effects of preceding light periods, etc., would operate in the direction of a smaller third dose reaction rate compared with the first dose rate, there is certainly a clear-cut inferiority in light and/or limiting dark reaction rates of a Janus Green B Hill reaction as compared with a Hill's solution Hill reaction.

The second result (b) comes from "double dose" experiments. In order that these experiments lead to conclusions about the reaction mechanism within the chloroplast, it is necessary to ascertain that the inhibitions observed are due to effects on light and/or limiting dark reactions within the chloroplast rather than to shading of the chloroplasts by dye. This can be accomplished by being sure that each type of reaction is occurring at its light saturation. Actually, as will be shown later, it is not necessary for the extraction of a meaningful conclusion that all the types of reactions involved be light saturated.

In these experiments, three kinds of Hill reaction runs were made at sufficiently high light intensities:

1. Quinone as the first dose of Hill oxidant. Call the initial rate in this run Q_1.

2. Janus Green B as the first dose and a second similar dose of Janus Green B. Call the initial rate from the first Janus Green B dose JGB_1, and the initial rate from the second Janus Green B dose JGB_2.

3. Janus Green B as the first dose, and a second dose of quinone similar to the quinone dose in the first kind of run. Call the initial rate of the quinone second dose Q_2.

One can express the second result (b) concisely in the form $JGB_1/JGB_2 > Q_1/Q_2$, where it is supposed that all rates are light saturated. If one achieved this result experimentally with only the information assur-

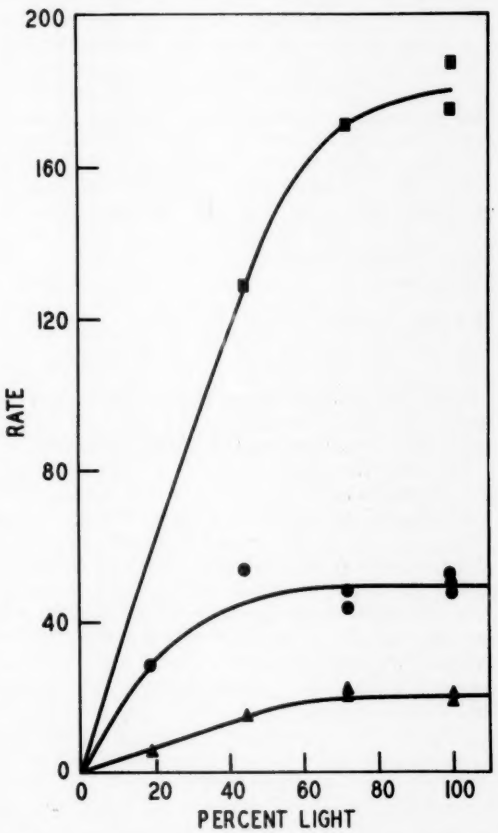


FIG. 4. Light saturation curves for first dose of quinone, first dose of Janus Green B, and second dose of Janus Green B. Abscissa is relative light intensity. Ordinate is rate of oxygen production in arbitrary units. Squares are for the rate with quinone; closed circles for the rate with the first dose of Janus Green B; triangles are for the rate with the second dose of Janus Green B.

ing one that Q_1 and JGB_2 were light saturated, it would still be safe to conclude that the same result would be obtained were all the rates light saturated. As is shown in figure 4, saturation was certainly achieved for all rates except possibly Q_2.

Five ml of spinach chloroplast suspension, taken from storage in dry ice and freshly thawed, containing 0.25 mg of chlorophyll and held at 20.6° C, were used per vessel, with 0.5 ml doses of Janus Green B containing 0.15 mg of dye, and 0.5 ml doses of quinone containing 0.61 mg of quinone in 0.01 *N* H_2SO_4. To obtain the saturation curves, calibrated screens were interposed between the lights and the experimental vessel, and the same relative light intensities for each type of reaction plotted in figure 4 correspond to the same absolute light intensities. In a single double-dose run, with the same light intensity during the reaction of both doses, data for this intensity could be obtained for both the first and second dose Janus Green B saturation curves. Since there was no noticeable dependence of yield from the first dose on light intensity in the fivefold light intensity range covered, and the complete yield could always be achieved in the first dose light period of seven to eight minutes, it was supposed that first dose light intensity, insofar as it was varied, was not significant in determining the rate for the second dose. When chloroplasts alone are illuminated, there is a small photooxidative oxygen uptake which is negligible compared with the Hill reaction oxygen production.

The results from this series of runs shows that

$$\begin{aligned} &50.1 \text{ mm. per min}/20.4 \text{ mm per min} \\ &\quad = JGB_1/JGB_2 > Q_1/Q_2 \\ &\quad = 181 \text{ mm per min}/150 \text{ mm per min.} \end{aligned}$$

Another similar series was also run using Hill's solution rather than quinone, and 5 ml of the spinach chloroplast suspension contained 0.28 mg of chlorophyll. The data in this series did not give such clear-cut evidence that light saturation had been attained for JGB_2 and for the first dose rate with Hill's solution (called HS_1; the second dose rate with Hill's solution is called HS_2), although light saturation for JGB_1 was clearly attained. Nevertheless, a similar situation seems to hold with Hill's solution as with quinone, and, if one lends a reasonable interpretation to the data, it appears that

$$\begin{aligned} &64.8 \text{ mm per min}/21 \text{ mm per min} \\ &\quad = JGB_1/JGB_2 > HS_1/HS_2 \\ &\quad = 147 \text{ mm per min}/145 \text{ mm per min.} \end{aligned}$$

Discussion

Since the Janus Green B used in all experiments reported in this paper had a high percentage of impurity, there is a certain amount of ambiguity in drawing detailed conclusions. However, the main results are mostly unaffected. Color change, data on reaction stoichiometry and data from light absorption spectra indicate that Janus Green B certainly is reduced. Therefore the conclusions from the data comparing the Janus Green B Hill Reaction to other Hill Reactions are probably unaffected by the presence of impurity, though an impurity could easily be the cause of the differences in rate between a Janus Green B Hill Reaction and the other Hill Reactions.

Summary

1. Janus Green B is reduced in a Hill-type reaction with illuminated chloroplasts.

2. The percentage yield of oxygen from this reaction falls off with increased initial concentration of dye. The curve of absolute yield of oxygen against initial dye concentration exhibits a maximum. "Double dose" experiments indicate that this peculiar behavior is due to the establishment of a "steady state" wherein dye reduction is counterbalanced by oxidation of reduced dye.

3. For a given light intensity at the chloroplast and with other factors similar, Hill's solution can cause a far faster oxygen evolution from illuminated chloroplasts than can Janus Green D.

4. The inhibition of oxygen evolution from illuminated chloroplasts which a first dose of Janus Green B exercises over a subsequent dose of Janus Green B is percentagewise far greater than the inhibition over a subsequent dose of Hill's solution or quinone.

The author wishes to thank Dr. Allan H. Brown for much helpful advice and discussion throughout the course of this work, and for his assistance in running the mass spectrometer experiments.

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PHOTOCONTROL OF LEPIDIUM SEED GERMINATION[1]

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The same reversible photoreaction has been found to control seed germination of some lettuce (*Lactuca sativa* L.) varieties and floral initiation of *Xanthium saccharatum* Wallr. (1, 2). Observations are extended here to the light response of seed germination of *Lepidium vriginicum* L. and *L. densiflorum* Schrad. Seed of the former had earlier been noted to be light-sensitive (10). The results permit a closer definition of the reversible photoreaction and give some knowledge about the immediate reactions with which it is associated. A better understanding of the manner of action of several factors such as varying temperature and presence of nitrate or coumarin, known to affect germination of some seeds, is afforded.

METHODS AND PRELIMINARY OBSERVATIONS

Methods used were similar to those described for lettuce seed (3). In short, seeds were placed in glass Petri or square plastic dishes on blotters that had been wetted with water or solutions. They were held in darkness between black cloths in germination chambers at definite controlled temperatures for various periods and then subjected to the experimental treatment such as irradiation and shift of temperature. Three or more days after treatment the germinated seeds were counted. Irradiation was with either incandescent or fluorescent lamp sources equipped with filters to give radiation in the desired regions or with the spectrograph previously described and used in studies of photoperiodic responses (8). When the spectrograph was used the spectrum was reflected downward by a mirror and dishes containing seeds were placed at various positions in the spectrum for various periods as required.

A mixture of seeds of the two Lepidium species was collected locally and increased in the greenhouse. Here the two species were separated on the basis of leaf shape, type of inflorescence, and orientation of embryo within the seed. Lots of seeds of each species were collected and held in an icebox at about 5° C prior to use which extended over about 1 year. Preliminary experiments were performed to determine temperatures, times of imbibition, effective concentrations of solutions and other factors necessary for design of experiments.

Initial populations of 400 seeds in 4 lots of 100 each were used for each individual measurement unless otherwise stated. Precision was accordingly low for lots with less than about 10 % germination or greater than about 90 %. Values were transformed through the use of probits (5) plotted against log energy.

Preliminary experiments indicated that seeds of *Lepidium densiflorum* resembled those of *Lactuca sativa* in their irradiation requirements for promotion and inhibition of germination. They changed in sensitivity to radiation, however, over a 4-month period after harvest and their germination in the dark at 15° C increased to more than 50 %. For these reasons observations on germination of *Lepidium densiflorum* seeds were limited to crucial comparisons with *L. virginicum*.

THE ACTION SPECTRUM: Experiments with seeds of *Lepidium virginicum*, involving constant and alternating temperatures and presence or absence of KNO_3 solutions and light, were performed to find suitable germination conditions for subsequent action-spectrum and other experiments. Not a single seed germinated in any of these lots without irradiation. With standard cool white fluorescent lamps with a red filter, which gave radiation from 5800 to 7000 Å and a biological effect equivalent to that obtained with about one joule/cm² in the region 6300 to 6600 Å, germination was measured at constant temperatures of 15°, 20°, 25°, and 30° C and at alternations of 16 hours at 15° and 8 hours at 25° C. At constant temperatures maximum germination occurred at 20° C in the presence of KNO_3 solution (table I). With water germination was much greater with temperature alternations than with any constant temperature and when KNO_3 solutions were used it was rather close to 100 %.

The increased germination resulting from alternations of temperature between 15° and 25° C (table I)

[1] Received July 1, 1954.

TABLE I

GERMINATION OF LEPIDIUM VIRGINICUM SEEDS *

TEMP. °C	% GERMINATION **	
	WATER	KNO_3
15	21	84
20	32	80
25	33	60
30	0	0
15–25 †	90	97

* Germinated on blotters wetted with water or 0.2 % KNO_3 at various temperatures at a radiant energy of about 1 joule/cm² in the region 5800 to 7000 Å (irradiation for 1 hr with 1000 fc from a standard cool white fluorescent lamp with a red filter) at the end of the second day.

** 200 seeds per treatment. Under all conditions dark controls gave no germination.

† Alternating.

was attained by a single shift from 15° to 25° C when irradiation occurred at the time of the shift (table II). These results naturally raise a question as to the time required at 25° C for enhancement of germination to be realized after a shift from 15° C with irradiation at the time of the shift. After 0, 4, 8, 12, 16 and 24 hours at 25° C germination percentages were 10, 37, 53, 51, 63 and 63. The full effect was found to result from about 12 to 16 hours at the higher temperature.

The germination resulting from a range of energies of promotive or inhibitory radiation can be expressed graphically as straight lines by transformation of the values. Such lines are obtained if the probits (5) corresponding to the germination percentages are plotted on a linear scale against energy on a logarithmic scale (fig 1). These lines were used, as illustrated in the next paragraph and in the figure, to estimate energies for construction of action-spectrum curves expressing 50 % effect.

For measurement of the action spectrum seeds were placed along the spectrum for definite periods. Radiant energies thus were not constant for the various wave-length regions and various percentages of germination were observed. The energies and percentages of germination fix points in the probit-log-energy field. The estimated energy required to obtain 50 % germination, that is a probit of 5.0, is found by displacing each point to the level of a probit of 5 along a parallel to the lines shown in figure 1. The abscissa of this latter point gives the estimated energy. In each case an effort was made to give radiant energy that would result in about 50 % germination so that the energy conversion factor would not be great. For example, a given radiation treatment having a relative energy of 0.5 resulted in a germination of 31 %. This fixes the point a of figure 1. Displacement to 50 % parallel to the lines for promotion leads to the point b, which has an abscissa of 1.26. Accordingly, 50 % germination requires a relative energy of 1.26.

TABLE II

GERMINATION OF LEPIDIUM VIRGINICUM SEEDS *

TEMP. °C FIRST 2 DAYS	TEMP. °C 3RD–6TH DAY	% GERMINATION	
		IRRADIATED SEEDS	NON-IRRADIATED SEEDS
15	15	37	0
25	25	41	0
15	25	92	0
25	15	32	0

* Germinated on blotters with water as affected by temperature regimen at a radiant energy of about 1 joule/cm² in the region 5800 to 7000 Å at the end of the second day.

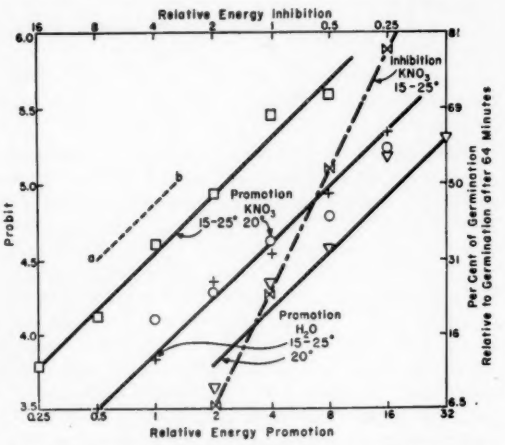

FIG. 1. Variation in germination of *Lepidium virginicum* seeds with radiant energy (fluorescent, 1000 fc red filter) at a constant temperature of 20° C and with a shift at time of irradiation from 15 to 25° C in presence of H_2O or 0.2 % KNO_3 solution. The longer broken line shows inhibition of germination at 7300–7500 Å. Broken line a–b illustrates method of reducing data described in text. Relative energies on abscissas correspond to exposures in minutes with about 3×10^{-4} watts effective irradiance.

This method of converting energies that gave observed germination percentages to those required for 50 % germination has been used to construct action-spectrum curves for promotion at wave lengths less than 6800 Å and for inhibition at wave lengths greater than 7250 Å (fig 2). Between these limits promotive and inhibitory effects are simultaneously involved to such a degree that attempts at conversion lead to ambiguous values. If very high energies are given at wave-length stations between 6800 and 7250 Å to either fully promoted or fully inhibited seed equilibrium values are obtained. These values range from appreciably less than 100 for wave lengths just above 6800 Å to values considerably greater than 0 for wave lengths shorter than 7250 Å. At some position between these limits an equilibrium value of 50 % occurs. For *Lepidium virginicum* the wave-length

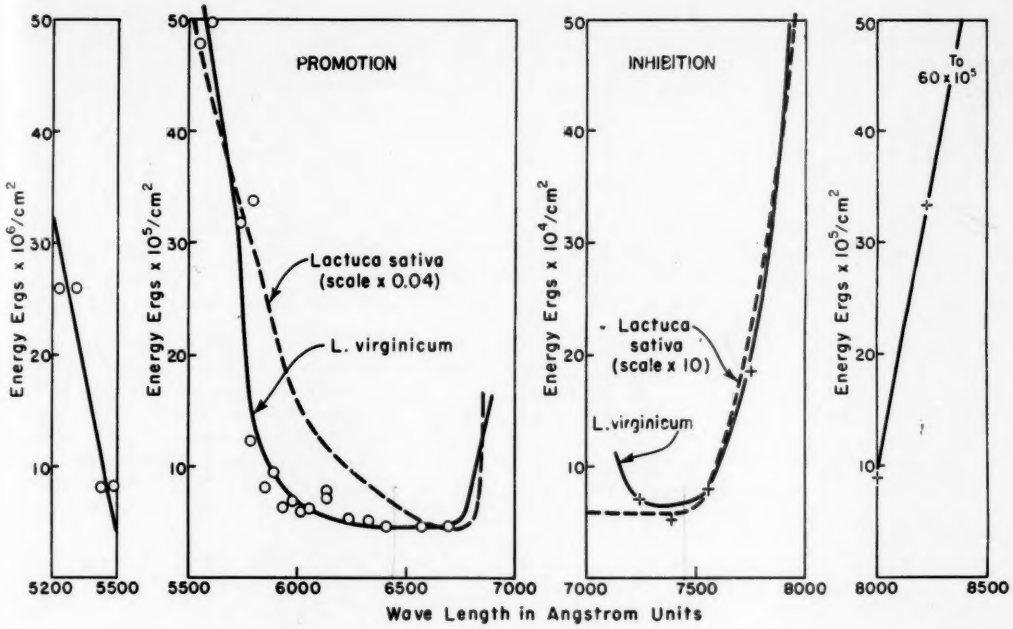

FIG. 2. Action spectra for promotion and inhibition to 50 % germination of *Lepidium virginicum* seeds at 20° C in 0.2 % KNO_3 solution (solid lines). Action spectra for promotion and inhibition of germination of seeds of *Lactuca sativa* shown for comparison.

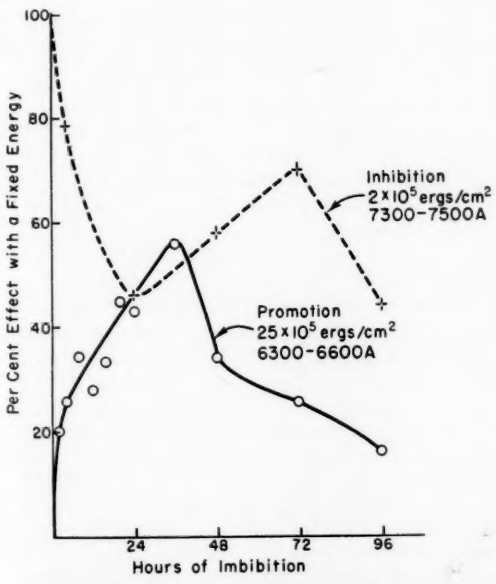

FIG. 3. Variation in sensitivity to promotion and inhibition of germination at 20° C of *Lepidium virginicum* seeds imbibed in 0.2 % KNO_3 solution with time of imbibition prior to irradiation.

region for the 50 % value of germination with 0.2 % KNO_3 at 20° C was between 6875 and 6900 Å.

Significant points were determined for *Lepidium densiflorum* seeds irradiated after 24 hours of imbibition in water at 15° C. Minimum energy for inhibition to 50 % germination was found at 7400 Å, the required incident energy under the particular conditions being 1.8×10^5 ergs/cm², and for promotion to

TABLE III

ENERGIES REQUIRED (ERGS $\times 10^5$/CM²) FOR 50 % GERMINATION OF LEPIDIUM VIRGINICUM AND L. DENSIFLORUM WHEN PROMOTED AT 6300 TO 6600 Å OR INHIBITED AT 7300 TO 7500 Å IN THE PRESENCE OF WATER OR SOLUTIONS

KIND OF SEED AND IMBIBED LIQUID	ENERGY FOR INHIBITION			ENERGY FOR PROMOTION		
	20° C	15° C	15–25° C *	20° C	15° C	15–25° C *
L. virginicum						
H_2O	0.3	...	0.4	14.0	...	5.8
0.2 % KNO_3	0.5	...	0.55	5.8	...	1.7
0.5 % $CS(NH_2)_2$	...	...	0.75	...	...	2.0
L. densiflorum						
H_2O	...	1.8	...	...	0.1	...

* Alternating.

50 % germination at 6650 Å, the required incident energy being 6.0×10^3 ergs/cm^2. The wave length for 50 % germination with very high energies was between 7000 and 7150 Å. Precision of these values was low because of the high germination of dark controls.

FACTORS CONTRIBUTING TO CHANGES IN SENSITIVITY OF SEEDS TO RED AND FAR-RED RADIATION: The energies required for promotion or inhibition of germination by a given amount depend on factors such as duration of imbibition at the time of irradiation, the presence in the water of such substances as coumarin, thiourea or potassium nitrate and occurrence of shifts in temperature during germination (fig 3 and tables III and IV). The radiant energies given for 50 % germination in table III were obtained from the probit-log-energy lines. The slopes and positions of these lines depend upon all the observed germination values between about 10 and 90 %. Every experimental result between these limits thus contributes to the precision of estimates of the energy required for a desired result such as 50 % germination.

The interaction of various energies in the red with coumarin concentration on the germination of seed of *Lepidium virginicum* at 15 to 25° C was physiologically significant. Low concentrations of coumarin resulted in a promotion of germination above that of control lots, while slightly higher concentrations of coumarin were inhibitory (table IV). Sensitivity of germination to inhibition by far-red radiation was also measured, but less minutely. Its change was the inverse of that for the red.

REVERSIBILITY: Germination of seed of *Lepidium densiflorum* and *L. virginicum* is repeatedly photoreversible by red and far-red radiation (table V). The red energy was the same for both, but the far-red energies differed somewhat. In the case of *L. virginicum* a 5 cm water filter was placed between the seed and the source to reduce heating of the seed during the 8-minute exposures.

DISCUSSION

Action spectra for promotion and inhibition of seed germination of *Lepidium virginicum*, *L. densiflorum* and *Lactuca sativa* (1) are closely similar on a relative scale. These action spectra are, moreover, closely similar to those for floral initiation (2), cuticle coloration in tomato (9), and internode and leaf elongation (4). The minimum energy requirement for action in one direction for each of these responses is at a wave length near 6400 Å and that for action in the opposite direction is near 7350 Å. The phenomena are certainly controlled by the same causal photoreaction.

Incident energy requirements for 50 % effect at the wave lengths for maximum effectiveness are shown in table VI for the several responses. The narrow range of energy values for the response to red and far-red radiation for germination of two of the three kinds of seeds, for floral initiation of Xanthium; and for cuticle coloration of tomato indicates that the growth-controlling reaction is operating at about the same level in many tissues.

The discussion can most readily be based on the scheme of reactions deduced from the results of seed germination and floral control by radiation.

This scheme is as follows:

(Photoreaction)

$$\text{Pigment} + \text{AR} \underset{7350\ \text{Å}}{\overset{6400\ \text{Å}}{\rightleftharpoons}} \text{Pigment-R} + \text{A}$$

$$+$$

$$\text{Molecule}$$

(Subsequent reaction) $\downarrow$

$$[\text{Pigment-R-Molecule}]$$

The photoreaction might be the reverse of the one indicated, that is, A might be on the left side and AR on the right.

One basis of the reaction scheme involves a feature of the response of a population to stimuli. This has been thought out particularly well in the probit analysis for insect toxicants, but has not been used widely in plant physiology (5). The population is formed of individuals of differing sensitivities, some very responsive and others insensitive to the stimuli. If two stimuli have different values for the mean response but the same variance then it is likely that the action of the stimuli has the same limiting factors. The variance is the reciprocal of the slope of the probit vs energy line. So if these lines are parallel for response

TABLE IV

EFFECT OF COUMARIN AT VARIOUS CONCENTRATIONS ON GERMINATION SENSITIVITY OF LEPIDIUM VIRGINICUM SEEDS TO RED RADIATION *

COUMARIN CONC. 10^{-5} MOLAL	% GERMINATION AFTER IRRADIATION WITH RED FOR MINUTES INDICATED					
	0	2	4	8	16	64
0.0	0	19	37	44	55	59
0.5	0	25	35	48	58	70
1.0	0	32	49	56	63	64
2.0	0	24	39	50	54	69
4.0	0	22	43	57	60	63
6.8	0	2	4	11	10	24
10.3	0	..	0.2	..	0.5	2.5

* Radiation about 0.3×10^{-4} watt effective.

TABLE V

GERMINATION OF LEPIDIUM DENSIFLORUM AND L. VIRGINICUM SEEDS IN DARKNESS FOLLOWING ALTERNATING FIXED RADIANT ENERGIES OF RED AND FAR-RED

NUMBER OF IRRADIATIONS		CHARACTER OF FINAL RADIATION	% GERMINATION	
RED	FAR-RED		L. DENSIFLORUM	L. VIRGINICUM
1	0	Red	92	45
1	1	Far red	22	0
8	7	Red	95	48
8	8	Far red	24	0
0	0	.....	8	0

to radiation in the presence of different stimuli the stimuli probably affect the same system at the same limiting point.

Thus, treatment of Lepidium seeds with 0.2 % KNO_3 solution has two distinct effects. First, it promotes total germination without change of variance of the population. In other words, the additional seeds that germinate in presence of KNO_3 and radiation are distributed in the same normal population for response to radiation as are those imbibed in water. Secondly, the mean radiant energy required for a given percentage germination of the seeds that can be influenced if saturating energies of this radiation are used is shifted. Thiourea has this same action. These results are obtained even though the concentrations of nitrate and thiourea used are such as to inhibit markedly seedling growth—presumably through reactions unrelated to the one being discussed.

If a factor such as imbibition in the presence of KNO_3 solution acts to change the sensitivity to red radiation, that to far red generally changes in the opposite direction. This can be seen from results in table III and from the change of sensitivity with time of imbibition prior to irradiation as shown in figure 3. A further fact bearing on the nature of the photoreaction is that the variances of the populations for promotion and inhibition of germination for *Lepidium virginicum* differ as shown by different slopes of the lines in figure 1.

To complete the argument, which can be done in several ways, the close agreement of results following one and eight reversals, as shown in table V, requires total pigment to be invariant during the course of the reversals. The coupled change in sensitivities then must be operating through other molecules than the pigment in the reversible photoreaction. These molecules are shown as AR and R, and this notation includes the possibility of R being hydrogen (H or 2H) or that AR is an electron donor and A an electron acceptor. Thiourea and nitrate affect the relative amounts of AR and A.

The absence of a temperature coefficient for the photoreaction requires AR and A to be non-limiting on the rate of the reaction. This indicates that they

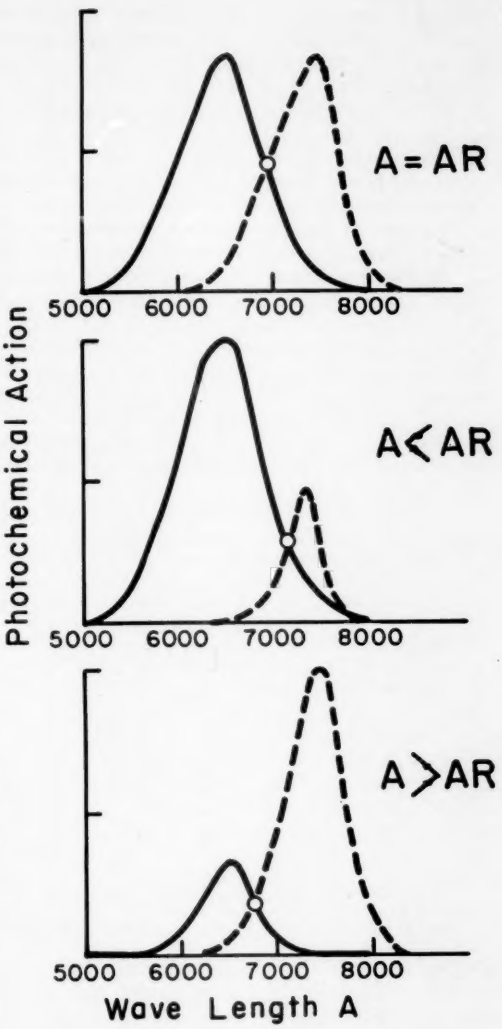

FIG. 4. A schematic diagram illustrating several relations between photochemical action in the red and far-red for the controlling photochemical reaction.

TABLE VI

INCIDENT ENERGIES REQUIRED FOR 50 % EFFECT BY PROMOTION AND INHIBITION FOR VARIOUS PLANT RESPONSES

RESPONSE	SPECIES	INCIDENT ENERGY (ERGS/CM^2 × 10^4)	
		RED	FAR-RED
Seed germination	*Lepidium virginicum*	140	3
	L. densiflorum	0.6	18
	Lactuca sativa	2	60
Floral initiation	*Xanthium saccharatum*	4	30
Cuticle coloration	*Lycopersicon esculentum*	2	..

are closely associated with the pigment. The photosensitivity in the two directions then depends upon the relative amounts of AR and A present. The consequences are shown in figure 4, which explains the fact that the wave lengths of greatest sensitivity are constant but that the wave length for 50 % response with high energy varies over a range of 300 Å. The possibilities illustrated in figure 4 are also in accord with the results listed in table VI. Thus, *L. virginicum* seeds correspond to A > AR, while for the other examples A < AR.

A similar reaction from photochemical literature is the reversible photoreduction of methylene blue or thionine in an anaerobic system in the presence of ascorbic acid, stannous chloride and other chemicals,

as recently reported (7). This, too, was shown to be repeatedly photoreversible, the effective wave lengths for reduction of the dye being in the visible and for its oxidation in the ultraviolet. The quantum efficiencies self-evidently depend upon the concentration of ascorbic acid although this dependence was not examined. Essentially the excited dye reacts with the reductant to form the leuco base and the excited leuco base reacts with the oxidant to regenerate the dye.

The question has existed as to whether the right or the left side of the photoreaction is further coupled with biological response. That it is the right side, the condition resulting from the action of red radiation, is shown by failure of the inhibition of flowering response in *Xanthium saccharatum* to be reversed after this condition has been established for 30 minutes by an irradiance in the red as shown in table VII taken from the thesis of Downs (4). Germination of light-sensitive seed is reversible in either direction for at least 4 hours which fact is indicative of another factor

TABLE VII

EFFECT OF TEMPERATURE DURING INTERVAL BETWEEN IRRADIATIONS ON LOSS OF REVERSIBILITY FOR FLORAL INITIATION OF XANTHIUM SACCHARATUM

INTERVAL BETWEEN RED AND FAR-RED RADIATIONS	FLOWERING CONDITION * WITH VARIOUS TEMPERATURES DURING THE INTERVAL	
	20° C	4° C
min.		
0	7	..
20	4	6
30	2	4
40	1	4
60	0	3

* Flowering condition was estimated on a scale of 8 stages of primordium development at time of dissection of 4 plants in each group, stage 8 being most advanced.

limiting the rate of the subsequent reaction. It is probably this factor that is associated with loss of light sensitivity when the seed coat is disturbed. The factor could, for example, simply be spatial separation of the active molecule from the photoreaction system in seeds. It could also be the introduction of a molecule different from A or AR that reduces or oxidizes Pigment or Pigment-R in darkness.

As seen from the values in table IV, coumarin at low concentrations promotes germination, reaching a maximum effectiveness at a concentration of 1×10^{-5} molal and inhibits germination at higher concentrations. Indoleacetic acid also has its maximum effectiveness on elongation of a coleoptile segment at a concentration of 1.1×10^{-5} molal (6). Coumarin would appear to be functioning by "two-point attachment" to another molecule (6). Coumarin probably does not act on the reversible photoreaction because it changes the variance of the population for germination on irradiation. A different variance obtains at each concentration.

When the temperature of imbibed *L. virginicum* seeds is quickly raised to about 40° C the ratio of AR to R changes rapidly. After some minutes the seeds reverse their relative sensitivities to irradiations in the region of 6500 and 7400 Å. If imbibed seeds are irradiated enough for about 50 % germination, then the radiation from the sun or an incandescent filament at about 3000° K should at high irradiances suppress germination but as the seeds warm, germination should be enhanced. This is observed.

The subject of this photoreaction controlling growth is now at the point where progress would be greatly aided by answers to three questions. (1) What is the pigment? (2) With what does auxin combine to be physiologically effective? (3) What reaction is controlled by the [Pigment-R-Molecule] combination?

SUMMARY

Action spectra for germination control of *Lepidium virginicum* and *L. densiflorum* seeds were measured to establish features of the reversible photoreaction that is a general factor involved in growth of higher plants. The maximum sensitivity for inhibition is near 7400 Å as previously found for lettuce seed germination and other responses. The sensitivity decreases monotonously with wave length by about 100 fold from 7400 Å to 8500 Å. The maximum sensitivity for promotion is near 6400 Å and this decreases monotonously by 100 fold from 6400 Å to 5260 Å. The seeds were unresponsive to inhibition or promotion of germination by radiation of wave lengths between 4000 and 5260 Å at 50 fold the energy required for 50 % germination at 6400 Å.

Interactions of the photoreaction with a shift of temperature and with nitrate and thiourea, factors known to affect germination of light-responsive seed, were measured. These are coupled in such a way as to cause opposite changes in sensitivity for promotion and inhibition of germination by the photoreaction. The relative sensitivity for promotion and inhibition of germination of *L. virginicum* was varied by more than 15 fold. This indicates that a molecule other than the pigment is involved in the photoreaction.

Coumarin promotes germination of *L. virginicum* at concentrations of 0.5 and 1.0×10^{-5} molal and inhibits germination at higher concentrations. This indicates that it effects control by two-point attachment in a rate-limiting reaction for germination as does auxin for cell elongation. Germination was almost completely inhibited by 1.0×10^{-4} molal coumarin.

A single shift in temperature of imbibed seeds of *L. virginicum* from 15° to 25° C at the time of irradiation in the red enhanced the population for germination without change of variance. A shift from 25° to 15° C was without effect on germination.

Reversible control of floral initiation in *Xanthium saccharatum* with radiation in the region of 7400 Å can only be accomplished for a period of 30 minutes after irradiation in the region of 6500 Å. This indicates that the pigment in the form with an absorption

maxima near 7400 Å undergoes a reaction with another molecule in darkness.

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INHIBITION OF ION ABSORPTION AND RESPIRATION IN BARLEY ROOTS[1,2]

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Since most current theories of ion absorption postulate the existence of metabolically produced ion carriers (18), studies of the effect of respiratory inhibitors on ion absorption have assumed increasing significance. Machlis (13) found that cyanide, azide, malonate and iodoacetate inhibited bromide absorption by barley roots. Lundegårdh (10) inhibited chloride absorption in wheat roots with iodoacetate. He also inhibited chloride and nitrate absorption with fluoride. Lundegårdh (11) and Milthorpe and Robertson (16) inhibited salt absorption with cyanide. Weeks and Robertson (27) found salt absorption in carrot tissue to be sensitive to carbon monoxide and the inhibition to be photoreversible. Robertson et al (20) linked salt absorption in carrots to oxidative phosphorylation by inhibition of absorption with 2,4-dinitrophenol (DNP). The literature cited strongly suggests the involvement of the Krebs cycle in ion absorption.

Most of the studies of the effect of inhibitors on ion absorption have been carried out by investigating the effect on either anion absorption or total salt absorption as measured by the conductivity method (16). Few, if any, studies have been undertaken to investigate the effect of inhibitors on cation and anion absorption simultaneously.

Differences or similarities in the metabolism associated with the absorption of K and Br ions in excised barley roots were examined by studying the effect of various inhibitors on K and Br absorption and on respiration. This was carried out in order to determine how closely glycolytic and Krebs cycle activity could be linked to the two ion absorption mechanisms. The roles of terminal oxidases and of phosphorylation also were studied by the use of inhibitors.

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[2] This paper is based on work performed in part under contract No. AT-(11-1)-34 project 5, with the Atomic Energy Commission.

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Materials and Methods

All experiments were carried out with excised roots from 6-day-old barley seedlings (variety Atlas 46). Seedlings were grown in a very dilute nutrient solution in the dark as described earlier (8) with the omission of the peroxide treatment. The excised roots were rinsed in distilled water and were centrifuged for 5 minutes at $65 \times g$. Weighed portions were then placed into various solutions.

Except where indicated otherwise, the temperature of growth and experiment was 26° C. The temperature of the bath in which respiration was measured was 25.5° C. All solutions used for absorption studies were aerated by compressed air except as indicated.

For pretreatments, root to solution ratios of 3 to 5 gm/l were used. In KBr solutions, 2 gm/l were used. In Warburg vessels 0.5 gm of root material was placed into 2.5 ml of water.

Potassium salts were used throughout this work, both in pretreatments and in subsequent treatments. After each treatment, roots were rinsed and centrifuged before being weighed out for the next treatment.

At the conclusion of the experiment, the roots

after each treatment were rinsed in distilled water and either were stored at −18 ° C until lyophillized or they were dried overnight at 60° C.

Potassium was determined in ashed aliquots of the dried and ground root material either by precipitation as cobaltinitrite followed by ceric sulfate titration (7) or by flame spectrophotometry. Bromide was determined as described earlier (7). Respiration, i.e., oxygen uptake, was determined in a standard Warburg assembly as described by Umbreit et al (26) using distilled water. At the conclusion of the absorption experiments, respiration measurements were made for at least 30 minutes on samples of the treated roots.

Sugar was extracted with 80 % ethanol in a soxhlet extractor and total reducing sugar determined by the method of Hassid (6).

The inhibition data are presented as percentage of control. These were determined form the slopes of ion content—time equations obtained in KBr solution following inhibitor pretreatment or obtained in the presence of the inhibitor and KBr where there was no pretreatment. The slopes are based on 6 points in KBr solution in most cases and were determined by the method of least squares. In most of the experiments reported here, pretreatment with inhibitor alone was used to avoid ion competition effects. Since in most of the experiments described here, potassium was absorbed from solutions containing slowly absorbed anions, sulfate was used as a control in order to have the same concentration of K present together with a slowly absorbed anion. In other experiments with this root material, differences in the rate of K absorption due to differing accompanying non-toxic anions were less than 15 %. This included absorption in the presence of rapidly as well as slowly absorbed anions.

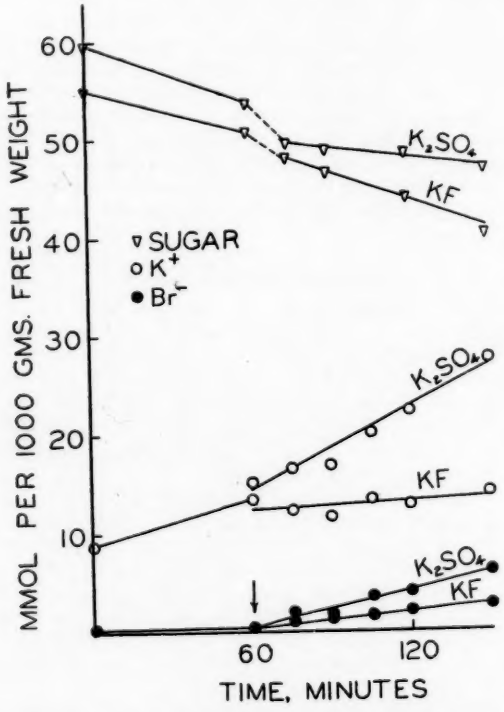

FIG. 1. Effect of 0.05 *N* KF pH 6.3 pretreatment and of 0.05 *N* K_2SO_4 pH 6.0 pretreatment, each followed by 0.005 *N* KBr pH 6.0, on total reducing sugar, K and Br content as function of time. Transfer indicated at arrow.

TABLE I

EFFECT OF INHIBITOR TREATMENT ON ION ABSORPTION AND RESPIRATION OF EXCISED BARLEY ROOTS

INHIBITOR	% OF CONTROL		
	K UPTAKE	Br UPTAKE	O_2 UPTAKE ††
0.02 *N* Fluoride *	53.0	53.1	65.8
0.04 *N* Fluoride *	3.0	40.0	40.5
0.0005 *N* Iodoacetate	63.5	69.8	...
0.001 *N* Iodoacetate	19.0	35.1	76.0
3×10^{-5} M Arsenite *	71.5	67.9	91.5
1×10^{-4} M Arsenite *	39.6	25.3	91.5
2×10^{-4} M Arsenite *	24.8	12.5	78.2
0.0025 *N* Fluoroacetate	49.2	51.6	76.2
0.005 *N* Fluoroacetate	28.3	36.0	69.2
0.01 *N* Fluoroacetate	17.1	40.9	58.8
0.01 M Trans-aconitate	68.0	66.6	...
0.02 M Trans-aconitate	37.2	53.3	60.0
0.005 M Malonate	60.7	58.2	70.0
0.01 M Malonate	8.4	29.7	45.0
0.001 M Cyanide *	63.5	50.5	70.5
0.002 M Cyanide *	26.2	26.0	64.8
90 % CO-10 % O_2 dark **	21.5	48.8	76.0
95 % CO-5 % O_2 dark **	−13.0 ‡	2.5	51.5
95 % CO-5 % O_2 light **	92.0	100.0	71.5
5.4×10^{-7} M Dinitrophenol **	40.5	61.3	...
1.1×10^{-6} M Dinitrophenol **	10.0	32.7	100.0
5.4×10^{-6} M Dinitrophenol **	−22.3 ‡	5.6	...
5×10^{-6} M Arsenate †	67.7	65.7	81.0
2×10^{-4} *N* Arsenate *	26.3	27.4	64.8

* pH 6.0 to 6.5.
** No pretreatment, inhibitor with KBr.
† pH 5.6.
†† Measured in distilled water at conclusion of experiment.
‡ Negative value indicates loss from roots.

RESULTS

FLUORIDE AND IODOACETATE: Inhibition of ion absorption was carried out by means of the glycolytic inhibitors, fluoride and iodoacetate. Roots were pretreated with 0.05 *N* KF or 0.05 *N* K_2SO_4 at pH 6 for 1 hour followed by 0.005 *N* KBr only at pH 6. Figure 1 shows that sugar utilization following fluoride pretreatment is not inhibited. On the other hand, potassium absorption is inhibited 92 % and bromide absorption is inhibited 57 %.

Table I shows the effect of various inhibitors on K absorption, Br absorption and oxygen consumption expressed as percent of control (the smaller the percent of control, the greater the degree of inhibition).

TABLE II

CHANGE IN CONTENT OF POTASSIUM AND BROMIDE OF 0.02 *N* POTASSIUM FLUORIDE TREATED ROOTS AS INFLUENCED BY INTERMEDIATE TREATMENT

INTERMEDIATE TREATMENT	MEQ/KG FRESH WT		
	ABSORPTION IN INTERMEDIATE SOLUTION	ABSORPTION IN 0.005 *N* KBR	
	K	K	BR
Pyruvate	3.9	0.2	5.3
L-Glutamate	7.8	0.6	6.2
Succinate	5.2	4.7	5.1
L-Malate	7.4	...	5.6
L-Glutamate + succinate	5.1	10.7	6.8
L-Glutamate + L-malate	4.6	2.6	6.2
Sulfate	3.7	3.6	7.0

Pretreatment: 0.02 *N* KF, pH 6.3, 2 hrs.
Intermediate Treatment: 0.02 *N* potassium salts, pH 4.5, 2 hrs.
Final Treatment: 0.005 *N* KBr, pH 4.55, 2 hrs.

All of the treatments listed are 2-hour pretreatments at pH 4.5 except as indicated in the footnotes. Following 0.02 *N* KF pretreatment, K absorption and Br absorption are inhibited equally and respiration is inhibited less than absorption. At the higher concentration (0.04 *N*), K absorption is inhibited much more than Br absorption and the inhibition of respiration is about the same as the inhibition of Br absorption.

Since Slater and Bonner (23) showed that fluoride can inhibit succinic dehydrogenase as well as enolase, bypassing a 0.02 *N* fluoride block was tried with pyruvate and other organic acids. Roots were pretreated with 0.02 *N* fluoride at pH 6.3 for 2 hours. The roots were then transferred for an additional 2 hours into solutions of 0.02 *N* potassium salts at pH 4.5 (intermediate treatment). This was followed by 2 hours in 0.005 *N* KBr at pH 4.5. The results given in table II indicate that glutamate and malate were quite effective in supporting K absorption during the intermediate treatment and that glutamate plus succinate supported the greatest K absorption subsequent to its use. The superior effect of sulfate compared to the other treatments with respect to Br absorption indicates further the divergence in behavior to inhibitors between the K and Br absorption mechanisms. In this experiment, glutamate was used in conjunction with other Krebs cycle acids to remove by transamination the oxalacetate which, if accumulated, would inhibit malic and succinic dehydrogenases. This technique was used by Swingle et al (25).

Table I shows the results of iodoacetate pretreatments on absorption and respiration. The results are similar to those with fluoride except that respiration is inhibited less. Experiments designed to overcome iodoacetate inhibition of absorption were carried out as with fluoride and a similar pattern of behavior was obtained.

ARSENITE: Arsenite, which inhibits electron transport from α-keto acids was tested. Table I shows that Br absorption is inhibited more than K absorption at all levels of inhibition. Respiration is only slightly affected.

FLUOROACETATE AND TRANS-ACONITATE: The use of fluoroacetate and trans-aconitate permits the Krebs cycle to be blocked at aconitase as shown by Buffa et al (1) and Saffran and Prado (21) respectively. Massey (14) later reported that trans-aconitate also inhibits fumarase.

The data of table I show that as the concentration of the fluoroacetate increases, the inhibition of K absorption increases more rapidly than the inhibition of Br absorption. Respiration is inhibited less than absorption.

Data obtained in experiments designed to overcome fluoroacetate inhibition using Krebs cycle acids are given in table III. During the intermediate treatment succinate induces the greatest absorption of K, followed by glutamate and fumarate. Citrate is relatively inactive in supporting absorption. Glutamate and malate exhibit some residual effect in the final period. The causes for the low immediate effect of malate and the subsequent fumarate effect as well as the relative ineffectiveness of citrate are not known.

Table I shows that trans-aconitate has an effect on absorption similar to fluoroacetate. An experiment designed to overcome the effects of trans-aconitate inhibition, carried out like that described in table III, produced a pattern similar to that obtained with fluoroacetate inhibition.

MALONATE: Table I demonstrates that 0.005 M malonate pretreatment produces equal inhibition of absorption of K and Br but that 0.01 M pretreatment causes K absorption to be inhibited more than Br absorption. Respiration is also inhibited at both concentrations.

Roots, pretreated with 0.01 M malonate, were subjected to either sulfate or succinate intermediate

TABLE III

CHANGE IN CONTENT OF POTASSIUM AND BROMIDE OF 0.01 *N* POTASSIUM FLUOROACETATE TREATED ROOTS AS INFLUENCED BY INTERMEDIATE TREATMENT

INTERMEDIATE TREATMENT	MEQ/KG FRESH WT		
	ABSORPTION IN INTERMEDIATE SOLUTION	ABSORPTION IN 0.005 *N* KBR	
	K	K	BR
Citrate	1.2	−0.1	3.6
L-Glutamate	4.2	2.3	4.4
Succinate	4.8	1.4	3.4
Fumarate	3.4	−2.0	2.9
L-Malate	0.8	3.3	4.0
Sulfate	1.0	1.4	3.5

Pretreatment: 0.01 *N* potassium fluoroacetate, pH 4.5, 2 hrs.
Intermediate Treatment: 0.02 *N* potassium salts, pH 4.5, 2 hrs.
Final Treatment: 0.005 *N* KBr, pH 4.80, 2 hrs.

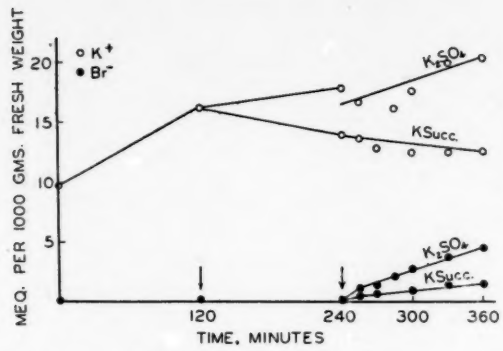

FIG. 2. Effect of 0.04 M K succinate treatment and c^e 0.03 N K_2SO_4 treatment, each followed by 0.005 N KBr, on K and Br content of 0.01 M K malonate pretreated roots as function of time. Roots removed from malonate at first arrow and placed in KBr at second arrow. All solutions single salt at pH 4.5.

treatment in an attempt to reverse inhibition by competition. Figure 2 shows a time curve of K and Br content in the period following such treatment. With 0.04 M succinate a definite enhancement of inhibition was noted, especially with reference to K absorption. Even in the final KBr solution, the roots lost potassium. Lower concentrations of succinate caused less deleterious effect than the higher concentrations of succinate (17). The results of treatments with other acids and a lower concentration of succinate during the intermediate period are summarized in table IV.

CYANIDE AND CARBON MONOXIDE: Machlis (13) and Lundegårdh (11, 12), among others, showed that potassium cyanide, which inhibits metallic oxidases, inhibited anion absorption while affecting respiration to a much less extent.

TABLE IV

CHANGE IN CONTENT OF POTASSIUM AND BROMIDE OF 0.01 M POTASSIUM MALONATE TREATED ROOTS AS INFLUENCED BY INTERMEDIATE TREATMENT

INTERMEDIATE TREATMENT	MEQ/KG FRESH WT		
	ABSORPTION IN INTERMEDIATE SOLUTION	ABSORPTION IN 0.005 N KBr	
	K	K	Br
Citrate	3.2	3.8	4.7
L-Glutamate	2.3	1.4	2.9
Succinate	2.3	3.0	3.2
Fumarate	5.1	3.4	4.7
L-Malate	6.6	3.7	5.3
Sulfate	4.5	1.7	4.6

Pretreatment: 0.01 M potassium malonate, pH 4.5, 2 hrs.

Intermediate Treatment: 0.02 N potassium salts, pH 4.5, 2 hrs.

Final Treatment: 0.005 N KBr, pH 4.65, 2 hrs.

Roots were pretreated wth cyanide at pH 6 for 2 hours. These pretreatments differed from the previous ones in several respects. Distilled water was aerated continuously in Pyrex bottles for 3 or 4 days prior to the experiment. Potassium cyanide was added and the pH was adjusted just before the roots were placed into the solution. After the roots were in position, the bottles were sealed and mixing was attained by inverting the bottle several times at 10-minute inervals for a 2-hour period. A the end of this time, the roots were treated as previously.

Table I shows that 0.001 M KCN causes a subsequent inhibition of bromide absorption which exceeds that of K absorption. At 0.002 M KCN the inhibitions are equal.

Experiments were carried out with CO in order to have a more specific inhibitor for cytochrome oxidase.

Carbon monoxide-oxygen and nitrogen-oxygen mixtures were prepared just before use by the evacuation technique of Umbreit et al (26). These experiments were not pretreatment types, i.e., the gas mixture was present with the KBr solution used. Portions of the gas-free solutions were displaced from stoppered bottles, after roots had been placed into them, by the desired gas. The sealed bottle was then placed in a shaker either in the dark or between banks of fluorescent lights. The lights, consisting of equal numbers of daylight and blue tubes, provided 400 fc at the outer face of the bottle and 250 fc at the inner face. Respiration measurements were made on roots in distilled water in Warburg vessels in the presence of these gases. For the illumination period of the respiration experiments, a 300-watt tungsten lamp provided 400 fc at the vessels and daylight and blue fluorescent lights provided 70 fc.

The results are given in table I. In the dark, 90 % CO caused K absorption to be inhibited more than Br absorption. With 95 % CO in the dark, K was lost from the roots. In the light, 95 % CO caused practically no inhibition of absorption of either ion.

Respiration data are given in figures 3 A and 3 B and table I. In the dark, 90 % CO caused a partial inhibition of respiration, but no inhibition in the light. In 95 % CO, reversal of respiratory inhibition by light was not complete, i.e., respiration was 72 % of the control.

2,4-DINITROPHENOL AND ARSENATE: The effect of phosphorylation uncoupling on ion absorption was studied by the use of DNP and KH_2AsO_4. Since the low concentration of DNP required for inhibition would rule out ionic competition during absorption, DNP was added at several concentrations to 0.005 N KBr at pH 4.5. The absorption and respiration data are presented in table I. At all concentrations, K absorption was affected more than Br absorption. At 5.4×10^{-6} M, K loss occurred and Br uptake was nil. At 1.1×10^{-6} M DNP, O_2 uptake was unaffected.

Arsenate pretreatments at 5×10^{-6} N at pH 6.1 and 2×10^{-6} N at pH 5.6 were carried out. Table I shows that arsenate inhibits subsequent K absorption

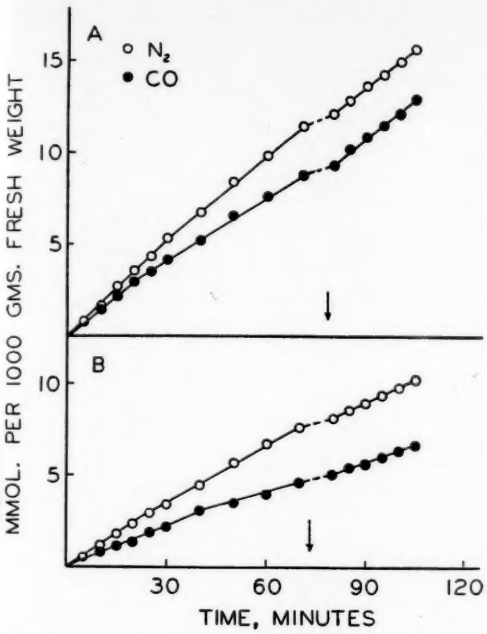

FIG. 3 A. Effect of 90 % CO-10% O_2 and of 90 % N-10 % O_2 on oxygen uptake in distilled water. Light turned on at arrow.

FIG. 3 B. Effect of 95 % CO-5 % O_2 and of 95 % N-5 % O_2 on oxygen uptake in distilled water. Light turned on at arrow.

and Br absorption equally. There is also some inhibition of respiration at both concentrations.

DISCUSSION

The inhibition of absorption by substances which inhibit glycolysis and the Krebs cycle, indicates that these pathways supply the intermediates or energy necessary for both potassium and bromide absorption in barley roots.

The fact that respiration is inhibited much less than absorption by iodoacetate, arsenite and fluoroacetate and lesser differentials are produced by fluoride trans-aconitate and malonate treatments, suggests three possible interpretations. There may be parallel sets of qualitatively similar enzyme systems which possess different properties or which occur at different sites in the cell. Secondly, there may be non-glycolytic and non-Krebs cycle pathways of respiration which cannot, however, support ion absorption. This is suggested in part by the observation that sugar metabolism following fluoride treatment appears to be non-glycolytic and non-aerobic in nature. Thirdly, there may be alternate pathways for respiration just over those steps which involve ion absorption. Probably all three alternatives exist.

Coxon (2) postulated multiple loci for enzymes. Millerd (15) showed in plant tissues that particulate sites, i.e., mitochondria, possess complete sets of the Krebs cycle enzymes. It is possible that some of these sites could be involved in ion absorption as well as other cell activities and some could be involved only in the latter.

The differential between inhibition of K absorption and inhibition of Br absorption also suggests a separation yet parallelism between the enzymes associated with the K and Br absorption mechanisms. At levels of inhibition between 50 and 90 %, (i.e., 50 to 10 % of control) fluoride, iodoacetate, fluoroacetate, transaconitate and malonate exert a greater effect on K absorption than on Br absorption. Arsenite, on the other hand, inhibits Br absorption more.

The relative ineffectiveness of pyruvate in overcoming fluoride inhibition of absorption may be due in part to a low activity of the pyruvate because of the high environmental pH in relation to the pyruvic acid pK of 2.5 (22). It was found that fluoride pretreatment inhibited the absorption as well as the metabolism of radioactive pyruvate (17). The closer proximity of the environmental pH to the pK of the Krebs cycle acids favor their activity and this may explain their greater degree of effectiveness in overcoming fluoride inhibition.

The effect of fluoride on ion absorption, therefore, is attributed primarily to the inhibition of enolase and secondarily to the inhibition of succinic dehydrogenase. The partial effectiveness of Krebs cycle acids in alleviating inhibition of absorption together with the common observation that anaerobiosis does not inhibit glycolysis but does inhibit absorption suggests that enolase is involved in absorption to the extent of controlling the rate of entry of substrates into the Krebs cycle.

Iodoacetate results are interpreted similarly except that the chief enzyme involved is probably triosephosphate dehydrogenase.

The results of the fluoride and iodoacetate inhibitions indicate that some step or steps of the Krebs cycle or closely associated reactions are essential to ion absorption.

The data on overcoming fluoroacetate and malonate inhibitions suggest that the bypassing of blocks at aconitase and succinic dehydrogenase can maintain absorption. The malonate studies indicate that succinate, which is quite effective in overcoming fluoride and fluoroacetate inhibitions and therefore is not toxic when metabolized, probably cannot displace malonate from succinic dehydrogenase and instead exerts a blocking action on other enzymes. The deleterious effect of glutamate may be due to interference with the normal utilization of α-ketoglutarate (19). Machlis (13) also reported that succinate failed to reverse malonate inhibition of respiration and had little effect on inhibited bromide absorption. The effectiveness of malate in overcoming malonate inhibition of absorption in the work reported here is presumably due to malate acting as a substrate for reactions which involve the absorption of ions. This suggests either that only certain series of steps are involved, i.e.,

those steps presumably free to react in common under both conditions of inhibition, or that several alternate sequences of steps can support absorption.

The inhibition of cytochrome oxidase by CO or KCN would result in blocking the Krebs cycle due to the linkage of succinic dehydrogenase to cytochrome oxidase. The observed inhibited respiration in 95 % CO in the light may be due to inhibition of copper enzymes (9) which apparently are not involved in ion uptake. The unequal inhibition of K and Br absorption by CO indicates that although the absorption of both ions is mediated via cytochrome oxidase, separate absorption mechanisms exist as previously indicated. The divergence in behavior between CN and CO on the relative inhibition of K and Br absorption may be due either (1) to the effect of CN not only on cytochrome oxidase but also on cytochrome c and other enzymes and compounds or (2) to the existence of the oxidase in two forms which possess different properties with respect to complexing with carbon monoxide and cyanide. The role of cytochrome oxidase may be primarily to govern the activity of the Krebs cycle rather than to act directly as ion carriers as suggested by Lundegårdh (12).

In addition to the more or less specific requirement for Krebs cycle activity, oxidative phosphorylation may participate in ion absorption as shown by the effect of DNP. Apparently the two absorption mechanisms, i.e., for K and for Br, are differentially sensitive to this substance. Substrate phosphorylation, as indicated by arsenate inhibition, also appears to participate in ion absorption. The lack of differential effect in this case suggests that the two sites are equally sensitive to arsenate or that the reaction affected occurs in common to both mechanisms.

The role of phosphorylation is of some interest since according to Goldacre (5) the major role of metabolism in absorption is to provide adenosinetriphosphate (ATP) which directly governs a postulated ion carrying activity of proteins. According to Lundegårdh (12) oxidative phosphorylation maintains the diffusion barrier in cells. Alternatively, ATP or similar compounds may be involved in ion absorption by, for example, reacting with intermediates of the Krebs cycle to form ion carriers or to destroy them. According to this view metabolism is needed both to provide ATP and to provide intermediates which interact to function as ion carriers. Steward and Street (24) speculated that phosphorylated energy-rich nitrogen compounds function as carriers. These are produced by oxidative metabolism.

If ions are carried by intermediate unstable compounds of the Krebs cycle, or if ATP is utilized during absorption, the act of absorption may stimulate the cycle. This would occur because in a sense the ions would be cofactors in oxidative metabolism. This may be a function of ions which may be otherwise physiologically inactive after being absorbed. There may exist alternate routes of metabolism which cannot support ion transport and perhaps such processes as growth, but can support cell maintenance.

The differing effects on K and Br absorption noted here may display different patterns in other plants or in barley with other ion pairs. The work of Epstein and Hagen (4) on cation competition and the work of Epstein (3) on anion competition during absorption suggest that ions may be bound at similar or different sites depending on the ionic species. If these sites have enzymes which are not identical in properties, differential effects by inhibitors should become apparent for them. On the other hand, ions bound at the same site should show identical behavior to inhibitors, provided similar enzymes are involved.

Summary

Pretreatment with potassium salts of fluoride, iodoacetate, transaconitate, fluoroacetate and malonate, inhibits subsequent K and Br absorption equally at less than 50 % inhibition. Pretreatment with these salts inhibits potassium absorption more than bromide absorption above this level.

Potassium arsenite pretreatment inhibits subsequent Br absorption slightly more than K absorption at all levels of inhibition.

Potassium cyanide pretreatment inhibits subsequent Br absorption more than K absorption at low levels of inhibition (more than 50 % of control) but inhibits both equally at 75 % (25 % of control).

When 2,4-DNP is added concomitantly with KBr, K absorption is inhibited more than Br absorption. Potassium arsenate pretreatment inhibits K and Br absorption equally at several levels.

Carbon monoxide (90 %) inhibits concomitant K absorption more than Br absorption in the dark. This inhibition of both K and Br absorption is almost completely reversed by light.

In all cases of inhibition except 95 % CO in the light, respiration was depressed less than ion absorption.

Various Krebs cycle acids can overcome fluoride and fluoroacetate inhibition. Malonate inhibition is enhanced by succinate but overcome by malate.

It is postulated that separate but parallel mechanisms exist for the absorption of K and Br ions.

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THE EFFECT OF DIFFERENT IRON AND MANGANESE NUTRIENT LEVELS ON THE CATALASE AND CYTOCHROME OXIDASE ACTIVITIES OF GREEN AND ALBINO SUNFLOWER LEAF TISSUES[1]

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From a physiological standpoint, it is difficult to assess separately the role of iron and manganese in plant nutrition because these elements have been shown to be mutually antagonistic. Shive et al (16, 20, 21) and Twyman (25, 26) have reported that a high nutrient level of manganese has a depressing effect on the absorption of iron from nutrient solutions and upon the maintenance of a high level of water-soluble iron in plant tissues, inducing symptoms of iron deficiency chlorosis. High nutrient levels of iron were likewise shown to have a depressing effect on the absorption of manganese from nutrient solutions and upon the maintenance of a high level of water-soluble manganese in plant tissues, although to a much lesser degree than the effect of manganese on iron. Oulette (14) found that the severity of manganese toxicity symptoms in soybeans decreased as the nutrient level of iron was increased in the substrate. Other instances of manganese-induced iron deficiency have been reported by Sideris and Young (18), Sideris (17) and Hewitt (8, 9, 10, 11) for several plants.

Sideris and Young (18) and Twyman (26) have suggested that manganese-induced iron deficiency in plants is a result of substitution of manganese for two hydrogen atoms or for iron in porphyrin molecules. In vitro substitution of iron by manganese in purified horseradish peroxidase with resulting feeble peroxidatic activity has been reported by Gjessing and Sum-

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ner (7). Theorell (23) reported that such a substitution eliminated all peroxidatic activity.

It has been reported that activities of the enzymes catalase and peroxidase are related to the presence in plant tissues of photoreceptive pigments such as chlorophyll (6) and flavines (5). Catalase seems to be involved in photosynthesis (6) and peroxidase in the light-activated indoleacetic acid oxidase system in peas (5).

To study the effect of different levels of iron and manganese nutrition on the activity of iron-containing enzymes of chlorophyll-deficient and chlorophyll-bearing tissues, albino as well as green plants were employed in the present studies.

Materials and Methods

In order to continue the active growth of chlorophyll-deficient leaf tissue during the experimental period albino scions were grafted into the stems of plants having green leaves. Green and albino seedlings of Russian sunflower (*Helianthus annuus* L.) seeds [2] which originated as a result of mutations induced by ultrasonic vibrations (29, 30), were used in these experiments. Green seedlings used for stock plants to which green and albino scions were grafted were siblings from an earlier generation of the originally treated seeds. Seeds for the green stock plants were sown on September 30, 1952, and seeds for the albino seedlings were sown 20 days later in white quartz sand. The normal seasonal daily sunlight period was extended to 16 hours with incandescent light. A minimum intensity of about 6 fc was provided.

Soon after the albino and green seedlings from the second sowing emerged from the sand, they were cleft-grafted, one per plant, into the apical portions of the stems of the older green sunflower stock plants. Both albino and green seedlings were grafted into the green stock plants in order to provide albino and green tissues which were produced under as similar conditions of growth as possible. During the period from November 5th to 13th, 55 albino and 34 green seedlings were grafted. Twenty-seven albino and 24 green grafts united successfully.

The grafted plants were placed in culture solutions on December 12th. The culture vessels were one-gallon wide-mouth glass jars, each containing two albino-grafted plants and one green-grafted plant. Composition of the nutrient solution used is given as follows: Macronutrient salts—0.001 M KH_2PO_4, 0.0045 M $Ca(NO_3)_2 \cdot 4\,H_2O$, 0.002 M $MgSO_4 \cdot 7\,H_2O$, 0.0015 M K_2SO_4, and 0.0013 M HNO_3; micronutrient elements—0.10 ppm Zn, 0.10 ppm B, 0.01 ppm Cu, and 0.01 ppm Mo. During the eight-day period prior to the initiation of the different iron and manganese nutrient levels, the concentrations of iron and manganese supplied was 0.20 ppm and 0.05 ppm, respectively, in all cultures. When the different nutrient treatments were started, concentrations of 0.001 ppm, 0.25 ppm, and 10.0 ppm were chosen as the low, adequate, and high levels of each element, respectively. Plants grown with a level of 0.25 ppm of both elements will be referred to as control plants. These nutrient levels together with the nutrient ratios of iron to manganese are given in table I. All nutrient salts were of reagent grade. Stock solutions of macronutrient salts were purified by the alkaline adsorption method of Stout and Arnon (22), followed by further purification with dithizone and carbon tetrachloride (15). All nutrient solutions were prepared with deionized water which had a specific resistance immediately after deionization of from 11 to 15 megohms.

Cultures receiving a nutrient level of either low iron, high manganese, or an adequate level of both elements were run in triplicate, and those receiving a nutrient level of high iron or of low manganese were run in duplicate. The nutrient solutions were renewed twice weekly by complete replacement.

Albino and green leaf tissues for catalase and cytochrome oxidase activity determinations were removed from the grafted scions at desired times after distinct leaf symptoms associated with different nutrient levels of iron and manganese became apparent. Activity determinations were carried out over a period of about 30 days. A final harvest of grafted scions was made on February 3, 1953. Aliquots of leaf tissue were dried in a forced-draft oven at 70° C for the

[2] Seeds were provided through the courtesy of Dr. Raymond H. Wallace, Department of Botany, University of Connecticut, Storrs, Connecticut.

Table I

Iron and Manganese Contents of Leaf Tissues of Green-grafted Scions of Russian Sunflower Plants Grown under Different Iron and Manganese Nutrient Levels

Nutrient level		Fe/Mn nutrient ratio	Tissue content *					
Fe	Mn		Water-sol. Fe	Water-insol. Fe	Total Fe	Water-sol. Mn	Water-insol. Mn	Total Mn
ppm	*ppm*		*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*
0.001	0.25	0.004	9.0	67.5	76.5	55.5	95.5	151.0
0.25	0.25	1.0	56.5	113.0	169.5	51.0	111.5	162.5
10.0	0.25	40.0	63.3	127.7	191.0	40.5	117.5	158.0
0.25	0.001	250.0	49.0	99.2	148.2	6.0	60.0	66.0
0.25	10.0	0.025	12.5	83.0	95.5	372.0	387.0	759.0

* Expressed on dry wt basis.

determination of total and percentage dry weights. Small aliquots of leaf tissue were freeze-dried for protein nitrogen determinations, and other aliquots were frozen for determination of water-soluble, water-insoluble, and total iron and total manganese contents.

Soluble iron and soluble manganese contents of frozen tissue samples were determined on the basis of sap expressed in an aluminum cylinder and piston under a pressure of 2000 lbs/in^2 applied by means of a Carver press (20). Digestion of samples and subsequent color development in the determination of iron and manganese were carried out according to the method of Toth et al (24).

Protein nitrogen was determined by extracting small samples of freeze-dried leaf tissues for 16 hours with a refluxing water-alcohol mixture (27). Extracted tissue was then wet-digested and the total insoluble nitrogen content was determined by the semi-micro Kjeldahl procedure.

Tissue samples were prepared for enzyme activity determinations by grinding one gram of young leaf blade tissue in a cold mortar and pestle with a small quantity of cold deionized water. Maceration was completed in a cold Ten Brock glass homogenizer. For cytochrome oxidase, the homogenate was then diluted 1 : 25 with ice-cold deionized water, and for catalase 1 : 100 with ice-cold 0.05 M phosphate buffer, pH 7.0. Just prior to use of the enzyme preparation for catalase activity measurements, an aliquot of the homogenate was diluted to an appropriate volume with the cold phosphate buffer.

Catalase activity was determined manometrically in a Warburg apparatus at 25° C in triplicate by a method similar to that described by Sizer (19). Each reaction vessel contained 2.0 ml of 0.10 M hydrogen peroxide made up in 0.05 M phosphate buffer, pH 7.0. The sidearm contained 1.0 ml of enzyme preparation. In each series of determinations, one reaction vessel received 1.0 ml of the phosphate buffer in the sidearm instead of enzyme; this reaction vessel served to measure the amount of spontaneous decomposition of hydrogen peroxide in the absence of catalase. Oxygen evolution was followed over a period of 5 minutes.

Cytochrome oxidase activity was determined spectrophotometrically by measuring the rate of oxidation of ferrocytochrome c at a wavelength of 550 mμ in a Beckman Model DU spectrophotometer (3, 28, 32). The reaction mixture was composed of 2.0 ml of 4.0×10^{-5} M ferrocytochrome c – 0.15 M phosphate buffer at pH 6.0, 0.9 ml of deionized water, and 0.10 ml of enzyme preparation, making a total volume of 3.0 ml. Each activity determination was carried out in triplicate.

Results

Growth Responses of Plants: Within 12 days after initiation of the different nutrient treatments, severe symptoms of iron-deficiency chlorosis were apparent on young green leaves of plants supplied with a low nutrient level of iron or with a high nutrient level of manganese. The striking similarity in leaf symptoms induced by these different treatments can be seen in figure 1. The only visual means of distinguishing between the symptoms of low iron and of high manganese on the plants as a whole was a brown color of the trichomes along the stems, petioles, and lower surfaces of older leaves of plants subjected to a high nutrient level of manganese. This symptom was not present on plants receiving a low nutrient level of iron or in any other nutrient treatment. Plants subjected to a high level of iron were somewhat stunted in growth as compared with the control plants. Leaves were small and dark green in color, and stems were spindling. Symptoms of manganese deficiency were evident on leaves of green plants grown with a low nutrient level of manganese as a yellowing of the youngest leaves and some interveinal yellow mottling of the older leaves. Control plants were large and well developed. There were no visual differences in the albino scions in the different nutrient treatments with the exception of the browning of

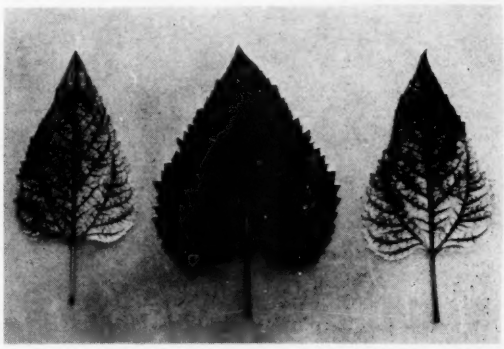

Fig. 1. Leaves of sunflower plants grown in culture solutions supplied with different iron and manganese nutrient levels. *Left.* Iron deficiency induced by a low nutrient level of iron. *Center.* Control. *Right.* Iron deficiency induced by a high nutrient level of manganese.

trichomes under conditions of high manganese already noted. Green scions attained heights of from 54 to 105 cm before harvesting, the average height being about 90 cm. Albino scions attained heights ranging from 9 to 50 cm with the majority averaging about 34 cm. Figure 2 shows albino grafts on green stock plants.

Iron and Manganese Contents of Plants: Results of chemical determinations of water-soluble, water-insoluble, and total iron and manganese contents of leaf tissues of green-grafted scions are shown in table II. No analyses were made for these elements in albino leaves because of the limited amount of tissue that was available.

As may be observed in table I, a low nutrient level of iron and a high nutrient level of manganese each resulted in very low water-soluble, and relatively low water-insoluble and total iron contents of the tissues as compared with the corresponding tissue fractions of iron with other nutrient treatments. With a low nutrient level of manganese, the water-

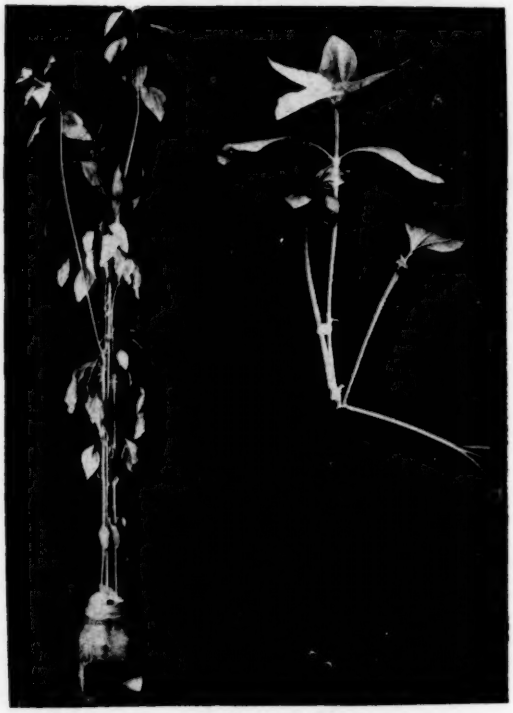

FIG. 2. Albino sunflower scions grafted to green sunflower plants. *Left*. Grown with a low nutrient level of iron. *Right*. Grown with control nutrient treatment.

soluble iron content (49.0 ppm) was much higher than with a high nutrient level of manganese (12.5 ppm). There were small, if any, differences between the water-soluble manganese contents of high iron, control, and low iron tissues, but with a high nutrient level of manganese water-soluble manganese was nearly 7 times higher (372.0 ppm) than in any other nutrient treatment. These results agree, in general, with those reported by Somers and Shive (20) for soybeans.

PROTEIN NITROGEN: The protein nitrogen content of albino and green leaf tissues grown with different levels of iron and manganese nutrition are shown in table II. In both green and albino tissues, the low nutrient level of iron resulted in relatively low protein nitrogen content. In green tissues, the high manganese treatment also resulted in a low protein nitrogen content but this was not the case in the albino tissues.

ENZYME ACTIVITY: Catalase and cytochrome oxidase activty data are shown in table II. Comparative determinations for nutrient treatments were carried out on the same day, but a series of replicate determinations for each treatment, run in triplicate, was carried out on different days over the 30-day harvest period. This was done to compare the relative activities under different light and other environmental conditions. Work to be reported in another paper has shown that catalase activity values of tissues grown with the same nutrient treatment varied considerably with differences in light intensity.

The data disclose that low levels of catalase and of cytochrome oxidase were found in both green and albino leaf tissues of plants grown with substrate levels of low iron as well as of high manganese. The highest activities of catalase were associated with substrate levels of both high iron and of low manganese. Enzyme activity of control plant tissues usually were intermediate in value. Catalase and cytochrome oxidase activities of albino tissues were usually less than those found for green tissues grown with the same nutrient treatments. In the case of low iron treatment there was no difference between the catalase activities of green and albino tissues.

TABLE II

PROTEIN NITROGEN CONTENT AND CATALASE AND CYTOCHROME OXIDASE ACTIVITIES OF LEAF TISSUES OF ALBINO- AND GREEN-GRAFTED SCIONS OF RUSSIAN SUNFLOWER PLANTS GROWN UNDER DIFFERENT IRON AND MANGANESE NUTRIENT LEVELS

NUTRIENT LEVEL		PROTEIN NITROGEN *		CYTOCHROME OXIDASE **		CATALASE ***	
FE	MN	ALBINO	GREEN	ALBINO †	GREEN ††	ALBINO ‡	GREEN ‡‡
ppm	*ppm*						
0.001	0.25	3.78	3.18	0.350	0.371	4.18	4.14
0.25	0.25	4.67	4.39	0.436	0.683	12.31	17.81
10.0	0.25	4.61	4.88	....	....	15.87	20.69
0.25	0.001	4.35	4.81	....	....	13.25	20.59
0.25	10.0	4.43	3.59	0.337	0.412	5.48	7.85

* Values expressed as % dry wt.
** Values expressed as K sec^{-1}; mg protein N in 3 ml.
*** Values expressed as ml O_2 evolved/min × mg protein N. Average of 5 (albino) and 4 (green) replicate determinations.
† L.S.D. (least significant difference) at 0.05 = 0.043 (differences not significant at 0.01).
†† L.S.D. at 0.05 = 0.153 (difference not significant at 0.01).
‡ L.S.D. at 0.05 = 6.93; at 0.01 = 9.50.
‡‡ L.S.D. at 0.05 = 3.03; at 0.01 = 4.25.

In both green and albino plants, average catalase activity of control leaf tissue was several times as great as those of low iron and of high manganese leaf tissues. Catalase activity of high iron leaf tissue was not significantly different from those of low manganese or of control tissues.

Cytochrome oxidase activity of albino leaf tissue of control plants was significantly higher than the activities of tissues grown with either low iron or high manganese. In the case of green tissues, cytochrome oxidase activity of controls was also significantly higher than that found for low iron or for high manganese.

Discussion

The results of this experiment substantiate the findings of Brown and Hendricks (2) that the nutrient deficiency of an element will be evident in a changed activity of enzymes requiring this element for function. This has also been shown by Nason et al (13). In accordance with this concept, low iron in the nutrient substrate resulted in a low activity of the iron-containing enzymes catalase and cytochrome oxidase in green-grafted and albino-grafted scions. Of perhaps more significance is the strikingly low activity of these enzymes in the presence of a high tissue content of manganese. The similarity in response of each enzyme to both low nutrient levels of iron and to high nutrient levels of manganese suggests that both treatments induce similar alterations in the metabolic pattern of these plants. This suggestion is substantiated by the similarity in the characteristic visual leaf symptoms, water-soluble iron, and in protein nitrogen contents of the leaf tissues. Furthermore, an earlier experiment with Havana Seed tobacco plants (31) demonstrated that the relative distribution of citric, malic, oxalic, and total organic acids of the leaf tissues was similar in low iron and in high manganese plants, and in high iron and in low manganese plants. The exact mechanism associated with these responses is not known. In the case of iron-containing enzymes, it is entirely plausible that in high manganese tissues there is direct competition between manganese and iron for a position in the heme nucleus of the enzyme as suggested by other workers (18, 26).

The inhibition of chlorophyll production in albino sunflower leaf tissues is not paralleled by the lack of production of the porphyrin nucleus for iron enzymes, since these enzymes are present in significant quantities. However, the relatively low catalase activity of albino tissues confirms the results published by Eyster (4) and Appleman (1) who found catalase activity of albino tissues to be less than that of green tissues. The presence of cytochrome oxidase in albino leaf tissues suggests that this enzyme mediates at least a portion of terminal respiration in these tissues. Definite proof of this, however, awaits a study of other terminal oxidases in these tissues.

From the information gathered in this and in previous experiments in this laboratory, it is concluded that on the basis of biochemical data presented and in the species of plants used, a high nutrient level of manganese in the presence of low iron induces a true iron deficiency. Thus, for each set of cultural conditions and for each nutrient level of iron, there is a nutrient level of manganese which will induce iron deficiency symptoms on many species of plants. If this level is surpassed, symptoms of manganese toxicity in addition to those of iron deficiency may become apparent. The browning of trichomes on sunflower plants observed in these experiments is believed to have resulted from a nutrient level of manganese which was higher than that required to induce only iron deficiency symptoms. It is recognized, however, that the general pattern of response to high manganese is not the same on all species of plants (8, 9, 10, 11, 12).

Summary

1. A solution culture experiment was carried out in order to study the effects of different iron and manganese nutrient levels on the water-soluble, water-insoluble, and total tissue content of both iron and of manganese, on protein nitrogen content, and on catalase and cytochrome oxidase activities of leaf tissues. Green and albino sunflower scions grafted to green stock sunflower plants were used.

2. Symptoms of iron deficiency chlorosis were evident on green portions of plants supplied with a high nutrient level of manganese, as well as with a low nutrient level of iron.

3. Symptoms of manganese deficiency chlorosis appeared on green plants supplied with a low nutrient level of manganese, but not on plants supplied with a high nutrient level of iron.

4. A low nutrient level of iron and a high nutrient level of manganese each resulted in a very low water-soluble iron content of green tissues as compared with controls.

5. In both green and albino tissues, the low nutrient level of iron resulted in a relatively low protein nitrogen content. In green tissues, the high manganese treatment also resulted in a low protein nitrogen content but this was not the case in the albino tissues.

6. Low levels of catalase and cytochrome oxidase activities were found in both green and in albino leaf tissues of plants grown with low nutrient levels of iron or of high nutrient levels of manganese.

7. Catalase and cytochrome oxidase activities of albino tissues were usually less than those found for corresponding green tissues grown with the same nutrient treatments.

8. Results support an hypothesis by other workers that induction of iron deficiency by high manganese may be due to a direct competition between manganese and iron for a position in the heme nucleus of the iron-containing enzymes.

9. It is concluded, on the basis of the biochemical data presented, that in the species of plant used, a high nutrient level of manganese in the presence of low iron induces a true iron deficiency.

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FREE AUXINS AND FREE TRYPTOPHANE IN THE STRAWBERRY[1,2]

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It has been previously demonstrated (15) that the growth of the strawberry receptacle is regulated by the achenes. This control is exerted through substances which are active in the Avena test, a standard test for auxins. The exact identity of these natural auxins, however, had not been investigated. The purpose of this paper is to report further experiments on the production of auxins by strawberry achenes, on the level of free tryptophane, a generally presumed raw material in the genesis of natural indolic auxins, and on the separation of the auxins of the strawberry by paper chromatography.

MATERIAL AND METHODS

Strawberries, var. Marshall,[3] were grown in pots in the greenhouse and hand pollinated. The fruits were removed at three day intervals during a period extending from 3 to 30 days after pollination, at which time the berries were ripe. Upon picking, the fruits were immediately brought to a cold room (5° C), the achenes were separated from the receptacles, both fractions were frozen by placing in a deep-freeze, and the frozen material was lyophilized. The lyophilized samples were used for auxin and tryptophane determinations.

Auxins were extracted with peroxide-free ether (water saturated) alone, or with a mixture of one part of acetone and two parts of ether (V/V) in an ice-bath at 0° C, in the dark, for 2 to 4 hours. It is generally agreed that such a procedure yields only "free" auxin, by contrast to treatments with alkali, proteolytic enzymes, or merely prolonged incubation at room temperature (29).

"Free" tryptophane was extracted according to the procedure of Dr. J. H. M. Henderson (unpublished) which consists of two steps: (1) the precipitation of proteins by boiling ethanol for 3 minutes, followed by the evaporation of the alcohol on a steam bath; (2) the extraction of tryptophane with hot water (repeated twice). The aqueous extract thus obtained was shaken with ether at pH 4.0 to purify it from possible traces of indole and anthranilic acid which give a positive test in the bioassay used. Then, the pH of the aqueous extract was adjusted to 6.0, and its tryptophane content measured quantitatively by the Lactobacillus assay (16, 17), a procedure which (in the absence of indole and anthranilic acid) is remarkably specific for L-tryptophane, as recently confirmed by Stowe (22).

The chromatographic techniques used here for auxins were similar to those of several workers in this field. Ascending chromatography on 2 cm wide strips of Whatman No. 1 paper was chosen; the solvent was that recommended by Stowe (22), i.e., isopropanol - 28 % ammonia - water (8:1:1, V/V), and the bioassay was that used by Kefford (8). An improvement of the latter technique was achieved as follows. A search made for hulless oats to avoid the tedious task of dehusking the thousands of seeds necessary for the bioassays revealed that at least two varieties, Laurel and Brighton, were suitable. The latter variety[4] was used throughout the course of this work. It was also tested in the standard Avena test in which the var. Victory has been used for years. The var. Brighton gave a satisfactory Avena test. For the straight growth bioassay, seeds were soaked for two hours in water and, then, laid down on wet facial tissue through which roots can grow freely (which is not the case with filter paper). The seeds were placed for about 4 hours at approximately 35 cm under a 32 watt G.E. white fluorescent "Circline" light filtered through a Corning filter No. 2404, to suppress growth of the mesocotyl. After the red light treatment, the seedlings were grown in a darkroom at 25° C and 85 % relative humidity. When the coleoptiles reached about 2.5 cm in length, the first 3 mm from the tip were cut off and discarded, while the next 4 mm were saved for the assay. Four mm rather than 10 mm sections were used to eliminate cells which had already elongated and to minimize geotropic curvatures (5). Sectioning was done with the van der Weij guillotine (26) which allows a precision of ± 0.1 mm. Only one 4 mm section was cut from each coleoptile. The primary leaf was left inside the sections to simplify the procedure and avoid injuring the coleoptiles (5). The sections were randomly distributed and floated on the solutions to be assayed. Kefford's technique was refined in two ways. Firstly, only 0.5 ml of total solution was used in each dish containing the section of the chromatogram. Such an amount of solution gives as good results as 1 ml (5). Secondly, in order to avoid growth variations due to pH effects, a phosphate-citrate buffer was used to maintain the pH of the solutions at 5.0. The composition of the medium was as follows: citric acid monohydrate (1.019 gm/l), K_2HPO_4 (1.794 gm/l), and sucrose (30 gm/l). Ten 4 mm sections were placed in each dish and left in the darkroom at 25° C for about 20 hours. They were then measured either with a plastic ruler to the nearest 0.5 mm or under a binocular with an ocular micrometer to the nearest 0.1 mm. Repeated measurements have shown that the accuracy and re-

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[3] Generous gift of the Tidewater Farms, Tidewater, Oregon.

[4] Kindly supplied by the Canadian Department of Agriculture, Central Experimental Farm, Ottawa, Canada.

producibility of this technique are satisfactory, the upper limit of the absolute error made on the mean of ten measurements being 0.25 mm with the plastic ruler and the standard deviation being ordinarily around 0.2 mm.

Results

The Free Auxin Content of Strawberry Achenes as Measured by the Avena Test: The ether extractable auxin after 2 to 4 hours at 0° C was measured quantitatively by the Avena test. From 10 to 100 mg (dry weight) of achenes were used on each extraction. The crude ether extracts were incorporated into agar blocks for the curvature test. At the same time, blocks with synthetic indole-3-acetic acid (IAA) were prepared with the two standard concentrations of 20 and 40 μg per liter. Care was taken to dilute each unknown extract to ascertain that the auxin concentration was within the proportionality range. The mean of several tests was used to establish the final figure of the auxin content.

The results of these tests are plotted in the graph shown in figure 1. Figure 1 concerns the achenes only, since no auxin can be extracted in this way

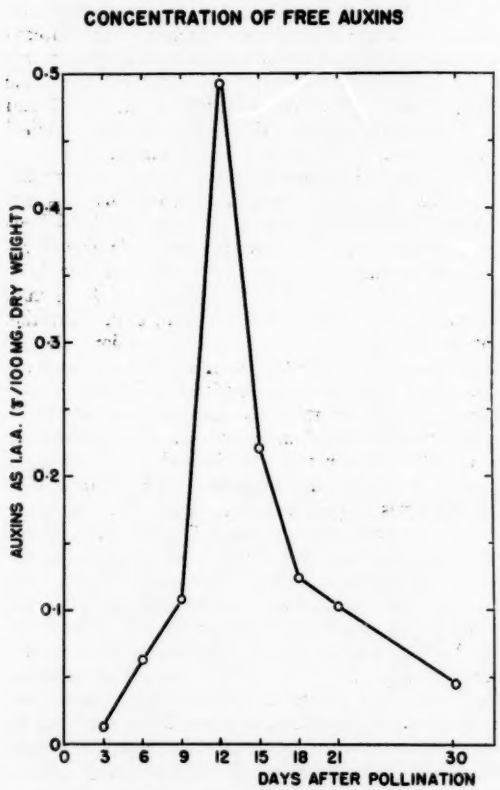

Fig. 1. Concentration of "free" auxins in the achene of the Marshall strawberry (measured by the standard Avena curvature test and expressed in IAA units).

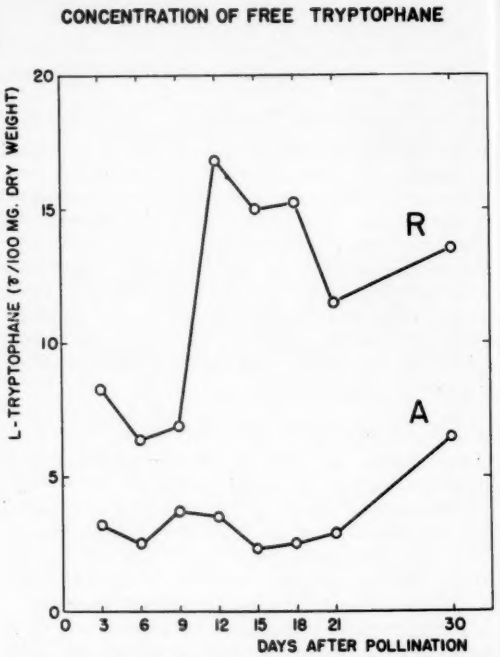

Fig. 2. Concentration of "free" L-tryptophane in the achenes (A) and the receptacles (R) of the Marshall strawberry.

from the receptacles (15). The prominent feature is the very steep peak occurring 12 days after pollination. It should be noted that the results are expressed on a concentration basis. If the total free auxin is computed per fruit, then the decline in the auxin content is less abrupt, since the weight of the achenes increases up to 30 days after pollination; but the sudden rise in the auxin level is just as steep. In the course of three days, the auxin concentration increases five fold, approaching the equivalent of 0.5 μg of IAA per 100 mg dry weight.

The Free Tryptophane Content of Strawberry Achenes and Receptacles: Preliminary determinations of free tryptophane in achenes and receptacles have been reported previously (16), but they concerned only three different stages of strawberry growth. A more complete survey has been made, using eight different samplings. The results, summarized by the graphs of figure 2, are presented on a concentration basis. At the first glance, one can see that the tryptophane level is higher in the receptacles than in the achenes. In fact, if the amount of free tryptophane per fruit is calculated, then the receptacle of a strawberry 12 days after pollination contains about 8 times more free tryptophane than all the achenes of the same fruit. In addition, it can be seen from the figure that the concentration of free tryptophane in the receptacles reaches a peak around 12 days after pollination, which is concomitant with the peak of auxin production in the achenes.

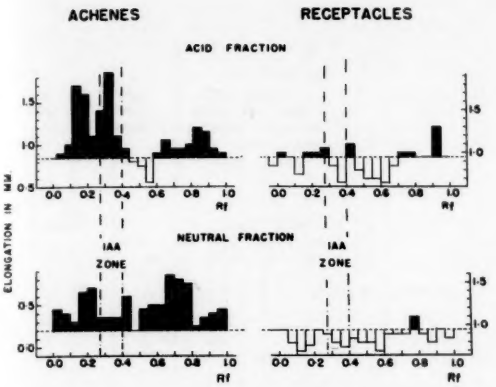

FIG. 3. Chromatographic separation of growth substances in achenes and receptacles of the Marshall strawberry, 12 days after pollination. (Note that the elongation scales do not start always at the same value. The dotted line represents the growth of the controls in each case. Black bars indicate growth promotion above growth of the controls, white bars growth inhibition. Variations in the order of 0.2 mm or less are not significant. 200–250 mg (dry wt) of material were extracted in each case.)

SEPARATION OF THE AUXINS OF THE STRAWBERRY: The Avena test which has been used both in a previous paper (15) and in the present one, to evaluate the amount of auxin in strawberry tissues, measures not so much the absolute amount of auxin as the balance between auxins and inhibitors. The negative results obtained previously with receptacle tissues may be due, perhaps, to the presence of an inhibitor masking an auxin which might, nevertheless, be there. Hence this first question: is there, in the receptacle, an inhibitor masking the presence of auxin or is there actually no auxin at all? A second problem follows: is there only one single auxin in the strawberry, or are there several?

In order to answer these questions, an attempt was made to separate the auxins and inhibitors present in the strawberry extracts by the use of paper chromatography. The results, shown on the diagrams of figure 3, show clearly that achene extracts, the neutral as well as the acid fraction, are growth promoting for coleoptiles sections as they are in the Avena test. On the other hand, extracts from receptacles show little or no growth promotion. Results of eight different chromatograms of extracts from receptacles have given erratic results with no consistent evidence for the presence of any particular auxin nor any particular inhibitor. In answer to the first question stated above, the results of the chromatograms agree with those of the Avena test and show that there is no appreciable free auxin nor inhibitor in the strawberry receptacles.

On the other hand, extracts of achenes have always been found to contain growth promoting substances. The second question as to whether there is one or several auxins in the achene extract can be easily answered. Figures 3, 4, and 5 demonstrate unquestionably that there are several auxins in the achene extract. The separation of these different auxins requires that the initial spot on the chromatogram is kept very small and that the chromatogram is cut into small sections (1 cm sections were used for a solvent front of 20 cm). It further requires that the extract has been sufficiently purified from fatty material which interfere with chromatography by not moving in the solvents used here and retaining a fraction of the auxins which are more soluble in fats than in aqueous solutions. To remove the fatty material, the technique of Jones and Riddick (7) was used. The ether extract was evaporated to dryness, then shaken with hexane and acetonitrile. Hexane removes fats while auxins of the IAA type remain in the acetonitrile. This fact was ascertained by shaking synthetic IAA, indole-3-acetonitrile (IAN) and indole-3-acetic ethyl ester (IAE) with hexane and acetonitrile: all three compounds remained in the acetonitrile fraction. After such a purification, the hexane was discarded, the acetonitrile fraction evaporated to dryness under reduced pressure, and the residue dissolved in a small volume of ether.

Another, even more effective purification technique was found to be a preliminary chromatography in glass-distilled water, the paper strips being equilibrated over the solvent for 17 hours before the actual run. When such a water chromatography is completed and the paper has been dried, the initial spot is cut off and discarded, and the rest of the chromatogram is re-extracted with ether. This extract, purified from colored material and fats, is then rechromatographed in the isopropanol-ammonia-water solvent. The total (acid + neutral) extract of 250 mg of achenes (12 days after pollination) corresponding to the diagram of figure 4, has been purified first by

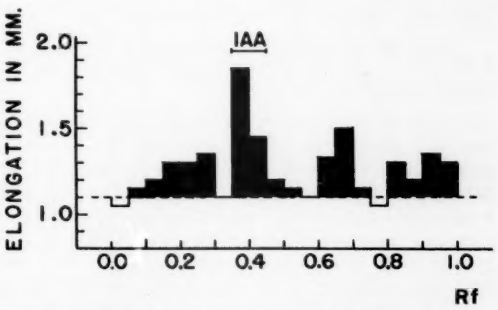

FIG. 4. Total extract of 250 mg (dry wt) of achenes (12 days after pollination) chromatographed in the isopropanol-ammonia-water solvent after purification through hexane and acetonitrile and a preliminary chromatography in distilled water. (The dotted line gives the growth of the controls. Black bars indicate growth promotion above controls. Each bar represents the mean of 10 sections. Differences under 0.2 mm are not significant.)

shaking with hexane and acetonitrile, then by preliminary chromatography in distilled water. Certain peaks are well separated.

Unfortunately, even then, certain auxins, especially near the ends of the chromatogram, are not well separated. For example, on the diagram of figure 4, it is hard to decide if the growth areas between Rfs 0.1 and 0.3, and Rfs 0.8 and 1.0 are due to one or more different substances. The differences between the growth responses are within the limits of the standard deviation from the mean. If, however, one pools the data of several different chromatograms, the picture becomes clearer. Although each individual experiment has its own errors and limitations, the successive growth peaks follow a common pattern. Figure 5 represents diagramatically the position of the various growth peaks of 11 different chromatograms of extracts of the same material (achenes, 12 days after pollination) in the same solvent. Although the extracts have been made in different ways and on different occasions (extending over a period of 5 months), one can recognize that certain peaks, even very inconspicuous ones, are found always in the same positions. They indicate the presence of several growth-promoting substances, of which the tentative list may be as follows:

Substance A: Rf around 0.1
Substance B: Rf around 0.23
Substance C: Rf around 0.35
Substance D: Rf around 0.5
Substance E: Rf around 0.7
Substance F: Rf around 0.83
Substance G: Rf around 0.95

There may be, possibly, another substance with Rf 0.6.

IDENTIFICATION OF THE AUXINS OF THE STRAWBERRY: The problem which we are facing now is the identification of the various substances which have been separated on the chromatogram.

Exposure to ultra-violet light failed to show any fluorescent compounds at the positions of the biologically active spots.

Color tests for indole compounds were more successful. One gram (dry weight) of 12-day-old achenes was extracted, and its acid fraction chromatographed. After spraying with a modified Salkowski reagent (4) diluted with an equal volume of ethanol, a definite pink spot (having the same shade of pink as synthetic IAA) appeared between Rfs 0.32 and 0.25, the center of the darkest area being located at Rf 0.29. The Rf of synthetic IAA chromatographed alone is 0.37, but it was found to be somewhat depressed by the addition of the achene extract. Another experiment, in which the extract of only 250 mg of the same material (acid fraction) was used, gave, with the Ehrlich reagent (2 % *p*-dimethylaminobenzaldehyde in 2 *N* HCl in 80 % alcohol), a purplish spot of the same color as synthetic IAA at the Rf 0.36. Thus, the Rf position and the color reactions with two different reagents agree in showing

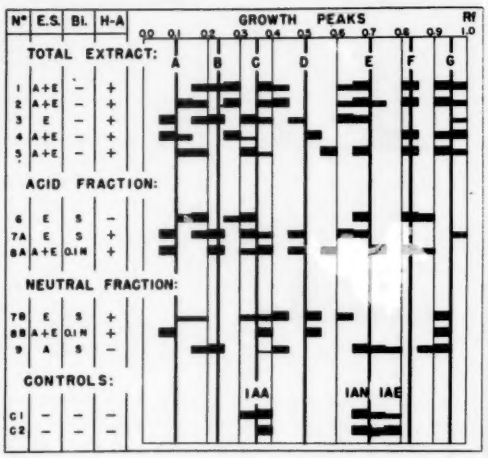

FIG. 5. The auxin spectrum in the strawberry achenes, 12 days after pollination (results of 11 different chromatograms in the isopropanol-ammonia-water solvent). *The 1st column* gives the number of the experiment: 7A and 7B mean that the same extract was used for the acid and the neutral fraction; C 1 gives the results of the chromatography of 0.1 microgram each of IAA, IAN and IAE using the bioassay; C 2 corresponds to the chromatography of IAA (2 μg), IAN (10 μg) and IAE (4 μg) in which the position of the auxins was located with the Ehrlich color reagent. *The 2nd column* gives the extracting solvent: ether (E), acetone (A). *The 3rd column* gives the concentration of the Na bicarbonate used: saturated (S) or 0.1 *N*. *The 4th column* indicates whether or not the extract has been purified by shaking with hexane and acetonitrile. *The location of the growth peaks* is represented as follows: high black rectangles show which segments of the chromatogram contain the maxima of the growth peaks; lower rectangles mark segments in which an appreciable fraction of growth (relative to the nearby peak) is located. The height of a rectangle is by no means proportional to the absolute amount of growth. The chart is only a qualitative one showing the position of the growth peaks, even the small ones.

that substance C, the most important quantatively of all the growth-promoting substances separated on the chromatogram, is IAA. From the size and the intensity of the colored spots, one could judge that the amount of IAA per gram of dry achene material was in the order of 1.5 to 2.0 micrograms. When measured by the Avena test, the total free auxin content of the same amount of material approaches the equivalent of 5 micrograms of IAA. This means that the natural IAA present in the achene extract accounts for only one-third to one-half of the growth-promoting activity. No other colored spot appeared; if other indole compounds were present, their amounts were too low to be detected colorimetrically.

In an effort to get a clue as to the identity of some of the other growth substances, chromatography

in other solvents was tried. Among about twenty different mixtures and combinations of solvents, distilled water was found to be the best, which confirms recent results by Sen and Leopold (19). Distilled water separates very well IAN and IAE which run very closely together in the isopropanol solvent. The paper strips were equilibrated overnight over the solvent as usual and double distilled water was used. Under these conditions, the Rfs which we consistently obtained were not those indicated by Sen and Leopold but the following ones: IAA (0.66), IAE (0.47), IAN (0.32). The extract of achenes 12 days after pollination, when chromatographed in water (after a preliminary chromatography in the isopropanol solvent), showed five growth peaks around the following Rfs: 0.15, 0.30, 0.45, 0.70, and 0.95. The second, third, and fourth may indicate the presence of IAN, IAE, and IAA in the extract.

Discussion

The data presented in figure 1 confirm the previous report on auxins in the strawberry (15), and substantiate the more general conclusion that the level of "free" auxins in developing fruits varies greatly from pollination to maturity. Such a variation has been reported in corn (1, 21, 30), in rye (6), in bean (14), and in the apple (10, 12). In most of these cases, a definite peak in the auxin concentration develops in the seeds at about mid-time between fertilization and maturation. The significance of this rapid rise is not completely elucidated, although it seems to be linked to events occurring in the endosperm. In the apple seed, in which Luckwill (10) described several peaks in auxin production, the same author noted a time correlation between the first and largest rise in the auxin level and the change of the endosperm from the free nuclear to the cellular stage. The second peak was concomitant with the development of a "secondary endosperm." It may be pertinent to mention here that cultures of corn endosperm in vitro produce exceptionally high amounts of IAA (1 mg per kg fresh weight), and indolepyruvic acid (22).

The role of free tryptophane as a precursor of auxins in the strawberry achene is not very clear. The concentration of this indolic amino acid in the receptacle, where apparently no auxin is generated, appears to be higher than in the achenes. Of course, the raw materials supplied by the leaves and the roots have to move through the receptacle to reach the achenes. It is possible that the tryptophane level in the achenes is low because of a constant drain on this amino acid to manufacture various compounds, among which would be the indolic auxins. Our knowledge on the genesis of these auxins is still confused. Tryptophane is probably not the immediate precursor of an auxin such as IAA. Several intermediates, including indole-pyruvic acid (IPA), recently demonstrated in corn kernels by Stowe and Thimann (23), may exist between the amino acid and the auxin.

To be able to draw a clear picture of the genesis of auxins, one should know their chemical identity. In the present work, a variety of growth-promoting substances present in the extract of 12-day-old strawberry achenes has been separated. The identification of these substances, however, has not always been possible. Only one substance, substance C, has been identified with IAA with reasonable certainty. Among the others, some are completely unknown, some have Rfs and biological properties similar to known auxins. The tentative list is as follows:

Substance A (Rf around 0.1): unknown.
Substance B (Rf around 0.23): possibly IPA.
Substance C (Rf around 0.35): IAA.
Substance D (Rf around 0.5): unknown.
Substance E (Rf around 0.7): possibly IAN with, perhaps, IAE (as indicated by the results of chromatography in water).
Substance F (Rf around 0.83): unknown.
Substance G (Rf around 0.95): unknown.

It may be seen from figure 5 that substances A, B, and C are generally found in the acid fraction, although traces of C (IAA) seem also to be present in the neutral fraction. Substance D may be found in both acid and neutral fractions. Substances E and F generally remain in the acid fraction, whereas substance G seems to move to the neutral one. The fact that E is carried from an ether solution into a bicarbonate aqueous solution does not preclude the fact that it might be IAN which, even at low concentrations, is very active on the straight growth test (3). The same phenomenon has been observed by Bennet-Clark and Kefford (2).

The presence in plant extracts of auxins different from IAA has been reported by many workers. For example, a growth-stimulating substance moving more slowly than IAA in an isopropanol-ammonia-water solvent has been reported by Kefford (8) in broad beans, Lexander (9) in wheat roots, von Denffer et al (27) in brussels sprouts, Weller et al (28) in cauliflower, Stowe and Thimann (23) in corn kernels. The latter authors identified their spot as being due to IPA, which gives a crimson color with the Salkowski reagent. Auxins moving faster than IAA in solvents similar to the isopropanol-ammonia-water one used in the present investigation have also been found in diverse materials: apple seed (11), crab-apple leaves (5), wheat roots (9), sweet corn kernels (24), cultures of Jerusalem artichoke (20), etc. The auxin in apple seeds found by Luckwill to have a Rf in the neighborhood of 0.83 has been identified by Teubner (25) as IAE, a natural auxin previously extracted from immature corn kernels by Redeman et al (18). The two substances extracted by Stowe and Thimann (24) from sweet corn and having the Rfs of 0.52 and 0.83 gave a positive Salkowski color test for indole compounds, but have not been identified. Neither have those in crab-apple leaves, wheat roots and Jerusalem artichoke cultures.

Thus, the presence in the strawberry of auxins

other than IAA is not an isolated fact. A few authors, especially Luckwill (13) and Stowe (22) have reported four different auxins in the same plant extract; the diagram given by Lexander (9) for wheat root extracts shows five growth peaks. In the present case, however, as many as seven different growth substances have been detected in one single organ. One must consider, however, that a strawberry achene is a complex of many different tissues: embryo, endosperm, nucellus, seed coat and carpellar tissue all are present 12 days after pollination. The complexity of tissues may be reflected in the variety of substances detected. One must bear in mind also that some of these various compounds may represent breakdown products of one initial substance. Finally, some of the compounds detected in the bioassay may only be auxin synergists, merely increasing the growth resulting from residual auxin present in the coleoptile tissues. With due regard to all these possible restrictions, the multiplicity of growth substances found in the present investigation may nevertheless indicate that not one, but a whole family of auxins are at work in plants, certain ones perhaps being transformed into others.

Summary

A more detailed quantitative evaluation of free auxins in strawberry achenes confirmed the existence of a very steep and narrow peak in auxin concentration around the 12th day after pollination. The level of free tryptophane in both achenes and receptacles has also been followed during the development of the fruit. Tryptophane concentration is higher in the receptacle than in the achenes. A peak in the level of free tryptophane in the receptacle occurs concomitantly with the maximum in free auxin concentration in the achenes. Chromatographic treatment of acid and neutral fractions, as well as of total extracts of both achenes and receptacles, separated up to seven different growth substances in the achene extracts. The most prominent of these substances has been indirectly identified with indole-3-acetic acid, while the exact chemical nature of the others is not yet established. No appreciable quantities of either auxins or inhibitors were detected in the ether extracts of receptacular tissues.

In the course of this work some technical improvements have been devised, especially in the purification of the plant extracts and in the bioassay.

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CHANGES IN NITROGEN FRACTIONS AND PROTEOLYTIC ENZYMES OF SOYBEAN PLANTS TREATED WITH 2,4-DICHLOROPHENOXYACETIC ACID [1,2]

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The pronounced effects of the growth regulator, 2,4-dichlorophenoxyacetic acid (2,4-D) on protein and other forms of nitrogen in plants have been noted by a considerable number of investigators. The authors have referred to some of these studies in reporting (7) a loss of protein nitrogen from the leaves and an accumulation of this nitrogen fraction in the stems and roots of soybean plants treated with 2,4-D.

Rebstock et al (11), working with red kidney bean seedlings after application of 2,4-D to the primary leaf, found greater proteolytic activity in 2,4-D treated than in control stems, but lower proteolytic activity in treated than in control leaves.

Studies of the effect of 2,4-D on certain other enzymes in plants have been summarized by Weintraub (17) and by Bonner and Bandurski (2).

In the experiment described below attempts were made to measure proteolytic activity in organs of plants in which the distribution of a number of nitrogen fractions was also studied before and after treatment of the plants with 2,4-D.

Growth of the plants in nutrient solutions from which nitrogen was omitted during and following treatment with 2,4-D made possible a study of the effects of treatment on nitrogen already absorbed and largely assimilated prior to exposure of the plants to 2,4-D.

Materials and Methods

Cultural Treatment: Solution culture techniques described by Shive and Robbins (13) were followed in general, with nutrient solutions renewed intermittently at decreasing intervals as the plants increased in size. Lincoln soybean seedlings, after germination at 30° C and a short period of growth while held upon plastic netting, were transferred to one-gallon culture jars, three plants to each jar. The basic nutrient solution used until the plants were 32 days old and more than 14 cm tall was essentially formula I of Shive and Robbins (13) with respect to macronutrient elements, supplemented with micronutrient elements as follows: 1 ppm Fe, 0.25 ppm B, 0.5 ppm Zn, and 0.5 ppm Mn. When the plants were 32 days old, the level of Mn and Zn in the solutions was reduced to 0.125 ppm of each. At this time all $(NH_4)_2SO_4$ was omitted from the solution, which was also modified to include the following concentrations of salts: 0.002 M KH_2PO_4, 0.0005 M K_2SO_4, 0.002 M KNO_3, 0.0045 M $Ca(NO_3)_2$, and 0.002 M $MgSO_4$. The initial pH of this solution was 4.7. Later, during and following exposure to 2,4-D, the plants were grown in a minus-N nutrient solution modified from the above by the substitution of 0.0045 M $CaSO_4$ for the $Ca(NO_3)_2$, by the omission of all KNO_3, and by an increase in the concentration of K_2SO_4 to 0.0015 M.

2,4-D Treatment: When the plants were 39 days old and from 38 to 40 cm tall with six fully expanded trifoliate leaves, they were treated with sodium 2,4-dichlorophenoxyacetate (2,4-D). Treated plants were exposed for 24.5 to 25 hours to 5 ppm acid equivalent of 2,4-D dissolved in the above-mentioned minus-N solution adjusted to pH 4.0. Control plants were transferred to the minus-N solution at pH 4.0 without 2,4-D. Omission of nitrogen from the treatment solution prevented further absorption of nitrogen during exposure of the roots to 2,4-D and also minimized pH changes. The low pH level in the solution was used because of evidence (5, 6) that a greater response was obtained to 2,4-D dissolved in solutions of low pH.

At the end of the one-day exposure period, plants were removed from the solutions, their roots were rinsed with distilled water, and the plants were placed in fresh minus-N nutrient solution without 2,4-D. Control plants were harvested at the start of 2,4-D treatment, and treated and control plants were har-

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vested one, three, and five days after 2,4-D introduction.

SAMPLING: The harvested plants were separated into leaf blades (leaves), stems plus petioles (referred to as stems) and roots and were dried in a forced draft oven at 70° C in preparation for analyses for nitrogen constituents or at 45° C for proteolytic enzyme assay.

Each tissue sample was a composite from 6 plants which represented two cultures of three plants each. Triplicate samples of this type were employed for each nitrogen analysis so that each average value reported was based upon tissue from 18 plants. Enzyme activity was measured in composite samples of a given tissue representing a total of nine plants. Determinations were repeated when questionable values were found.

NITROGEN CONSTITUENTS: Methods for the extraction of the soluble nitrogen fractions and for the determination of total, nitrate, and amide N were those formerly employed (7). Ammonium N was determined by a method used by Schlenker (12), amino N by Brown's modification (4) of Sörensen's formol titration. To accomplish this an aliquot of the hot water extract was brought to pH 8.0 with alkali and an equal volume of formaldehyde diluted 1:1 and adjusted to pH 5.0 was added, and the solution was titrated again to pH 8.0 with standard NaOH.

Peptide-N was determined by an enzyme hydrolysis method reported by Orcutt and Wilson (10) and Borsook and Dubnoff (3). An enzyme hydrolysis was preferred to an acid hydrolysis because of the demonstrated loss of amino N in humin formation by the latter method. A source of proteolytic enzymes for peptide hydrolysis was the fungus *Aspergillus wentii*.

To measure peptide-N, hot water extracts of plant tissue with equal aliquots of enzyme extract from the fungus were covered with toluene and incubated at 38° C overnight. The peptides were hydrolyzed to amino acids and the difference in amino N before and after hydrolysis served as a measure of peptide N. Amino N before and after enzyme hydrolysis was measured by the formol titration method.

PROTEASE DETERMINATIONS: The activity of the proteases was measured in glycerol extracts of tissues previously defatted in A.S.T.M. apparatus for 16 hours with ether. The tissues were then dried and the enzymes extracted according to methods reported by Blagowestschenski and Melamed (1) and Laufer et al (8). Five gm of the defatted tissues were mixed with 45 ml of 50 % glycerol and 5 drops of toluene and incubated at 30° C for 24 hours, with occasional shaking. After this, the mixtures were placed in muslin squares and pressed out into funnels containing No. 4 Whatman filter paper. The filtrates were then covered with 5 drops of toluene and placed in the refrigerator until further use. These enzyme extracts were clear amber solutions varying from light to dark shades.

To measure proteinase activity of the enzyme extracts, a 12 % gelatin solution was used as substrate. For peptidase activity, a 6 % peptone solution served as the substrate. Both substrates were adjusted to pH 7.0, the optimum pH for hydrolysis, and an equal volume of pH 7.0 citrate buffer was added. Equal volumes of enzyme solution and substrate were then mixed and after the addition of toluene were incucated at 37.5° C for 24 hours.

To estimate the activity of either one of these enzyme-substrate mixtures, a method described by Mounfield (9) was used. The procedure is a modification of the formol titration method, whereby the increase in carboxyl groups after enzyme hydrolysis of the substrate is used as a measure of the ability of the proteolytic enzyme to hydrolyze the peptide bonds. A 5-ml aliquot of the enzyme-substrate mixture was titrated before and after the 24-hr incubation period. The mixture was first titrated accurately to pH 8.5 with standard NaOH using a Beckman pH meter. Five ml of formaldehyde, adjusted to pH 7.0, were then added and the mixture was once more titrated to pH 8.5. The total titre of the enzyme-

TABLE I

PARTS OF SOYBEAN PLANTS HARVESTED AT INTERVALS AFTER TREATMENT WITH 2,4-D *

PLANT PARTS	DAYS HARVESTED AFTER TREATMENT						
	0	1		3		5	
	CONT **	CONT	2,4-D	CONT	2,4-D	CONT	2,4-D
	Dry wt in gm						
Leaves	2.67	2.80	2.96	2.71	2.60	3.57	2.58
Stems	1.81	2.21	2.26	2.16	2.21	3.03	2.63
Roots	1.25	1.33	1.29	1.33	1.37	1.45	1.34
Total plant	5.73	6.34	6.51	6.20	6.18	8.05	6.55
	% Dry matter						
Leaves	16.7	14.5	14.5	14.8	14.9	17.3	19.5
Stems	11.8	11.1	11.1	10.7	11.0	12.2	12.7
Roots	8.1	8.1	8.4	8.0	7.9	8.0	7.8

* Plants treated one day with 5 ppm 2,4-D in nutrient solution.
** No treatment with 2,4-D.

substrate mixture before incubation, subtracted from the total titre of the mixture after incubation, expressed in ml of 0.05 *N* NaOH then served as the measure of activity of the proteolytic enzymes under consideration. Total titre values, rather than titre after formaldehyde addition alone, were used because of evidence referred to by Mounfield (9) that total titre is probably a more reliable estimate of the total scission of the peptide linkages.

Experimental Results

Visible Plant Responses: Within 6 hours after 2,4-D treatment, twisting of stem tips and epinasty of petioles occurred. These symptoms were then followed by enlargement of stems and bases of petioles and whitening of the surface of stems within about three days. By the fifth day after treatment with 2,4-D, when the final harvest of plants was made, some wilting and slight drying of the first three trifoliate leaves and also of the topmost fully expanded trifoliate leaf was evident. The middle two or three trifoliate leaves, on the other hand, showed no external changes at this time. Treated plants also failed to form new leaves and to grow taller during the five-day interval following exposure to 2,4-D.

One extra culture of treated plants grown for two weeks after 2,4-D introduction had root initials along the surface of the lower third of the stems.

Dry Matter: No appreciable differences in total dry weight or percentage of dry matter (table I) were observed between controls and treated plants in the first or third day harvests. Not until the fifth day after 2,4-D introduction did the leaves of control plants show higher dry weights and a lower percentage of dry matter than those of treated plants. The

Table II

Nitrogen Fractions * of Soybean Plants Harvested at Intervals after Treatment with 2,4-D **

Nitrogen fractions	Days harvested after treatment						
	0	1		3		5	
	Cont †	Cont	2,4-D	Cont	2,4-D	Cont	2,4-D
			Leaves				
Total N	5.71	5.87	5.81	5.61	5.38	4.55	4.43
Organic N	5.58	5.79	5.75	5.51	5.30	4.53	4.37
Protein N	4.61	4.89	4.80	4.61	4.32	3.69	3.38
Sol. org. N	0.97	0.90	0.95	0.90	0.98	0.84	0.99
Amino N	0.24	0.21	0.23	0.17	0.22	0.18	0.24
Amide N	0.09	0.08	0.13	0.07	0.06	0.09	0.15
Peptide N	0.09	0.10	0.11	0.10	0.07	0.12	0.11
U.S.O. N ††	0.55	0.51	0.48	0.56	0.63	0.45	0.49
Nitrate N	0.13	0.07	0.06	0.08	0.08	0.02	0.03
Ammonium N	0.00	0.01	0.00	0.02	0.00	0.00	0.03
			Stems				
Total N	2.67	2.32	2.75	2.16	2.91	1.77	3.15
Organic N	2.10	1.93	2.41	1.74	2.64	1.56	2.92
Protein N	1.37	1.31	1.59	1.22	1.69	1.14	1.94
Sol. org. N	0.73	0.62	0.82	0.52	0.95	0.42	0.98
Amino N	0.26	0.19	0.22	0.16	0.24	0.10	0.25
Amide N	0.17	0.11	0.13	0.11	0.11	0.11	0.22
Peptide N	0.13	0.11	0.12	0.08	0.10	0.10	0.11
U.S.O. N ††	0.17	0.21	0.35	0.17	0.50	0.11	0.40
Nitrate N	0.56	0.39	0.33	0.40	0.26	0.18	0.17
Ammonium N	0.01	0.00	0.01	0.02	0.01	0.03	0.06
			Roots				
Total N	3.07	3.03	3.02	2.24	3.03	1.99	3.23
Organic N	2.53	2.84	2.76	2.12	2.89	1.93	3.15
Protein N	2.07	2.33	2.21	1.64	2.31	1.57	2.49
Sol. org. N	0.46	0.51	0.55	0.48	0.58	0.36	0.66
Amino N	0.17	0.13	0.14	0.09	0.13	0.08	0.16
Amide N	0.13	0.06	0.07	0.03	0.02	0.01	0.03
Peptide N	0.14	0.10	0.09	0.11	0.11	0.11	0.11
U.S.O. N ††	0.02	0.22	0.25	0.25	0.32	0.16	0.36
Nitrate N	0.54	0.19	0.25	0.09	0.12	0.03	0.06
Ammonium N	0.00	0.00	0.01	0.03	0.02	0.03	0.02

* Expressed as % dry wt of plant part.
** Plants treated one day with 5 ppm 2,4-D in nutrient solution.
† No treatment with 2,4-D.
†† Undetermined soluble organic N by difference.

small difference in percentage dry matter between treated and control plants on the fifth day indicates that most of the leaf tissue of treated plants was still not dehydrated.

No significant change in total dry weight of leaves or roots of treated plants occurred during the five days following exposure to 2,4-D in contrast with definite increases in dry weight of control leaves and roots. Although the dry weight of the stems of treated plants showed some increase by the fifth day after treatment, the dry weight of control stems showed a greater increase during the same interval.

NITROGEN FRACTIONS: By the third day after 2,4-D introduction several nitrogen fractions were markedly different in a given organ of treated plants from the respective fraction in the same organ of corresponding controls either on a percentage of dry weight basis (table II) or on a per plant basis (table III). In plants harvested two days later, these differences between treated and control plants were accentuated. Total, organic, and protein-N expressed on either basis were markedly higher in the stems and roots of treated plants than in corresponding controls three days after 2,4-D introduction. Further increases in these N fractions in stems or roots of treated plants by the fifth day resulted in even greater differences between treated plants harvested at this stage and control plants harvested at the same time or earlier.

Conversely, when expressed on either a percentage or a per plant basis, the treated leaves by the third day showed a decrease in total, organic, and protein N when compared to the corresponding controls harvested at the same time or earlier. Both concentrations and total amounts of these fractions in the leaves of treated plants harvested five days after

TABLE III

NITROGEN FRACTIONS * OF SOYBEAN PLANTS HARVESTED AT INTERVALS AFTER TREATMENT WITH 2,4-D **

NITROGEN FRACTIONS	DAYS HARVESTED AFTER TREATMENT						
	0	1		3		5	
	CONT †	CONT	2,4-D	CONT	2,4-D	CONT	2,4-D
				Leaves			
Total N	152	164	172	152	140	162	114
Organic N	149	162	170	149	138	162	113
Protein N	123	137	142	125	112	132	87
Sol. org. N	25.9	25.2	28.1	24.4	25.5	30.0	25.5
Amino N	6.4	5.9	6.8	4.6	5.7	6.4	6.2
Amide N	2.4	2.2	3.8	1.9	1.6	3.2	3.9
Peptide N	2.4	2.8	3.3	2.7	1.8	4.3	2.8
U.S.O. N ††	14.7	14.3	14.2	15.2	16.4	16.1	12.6
Nitrate N	3.5	2.0	1.8	2.2	2.1	0.7	0.8
Ammonium N	0.0	0.3	0.0	0.5	0.0	0.0	0.8
				Stems			
Total N	48.3	51.3	62.2	46.7	64.3	53.6	82.9
Organic N	38.0	42.7	54.5	37.6	58.3	47.3	76.8
Protein N	24.8	29.0	35.9	26.4	37.3	34.5	51.0
Sol. org. N	13.2	13.7	18.5	11.2	21.0	12.7	25.8
Amino N	4.7	4.2	5.0	3.5	5.3	3.0	6.6
Amide N	3.1	2.4	2.9	2.4	2.4	3.3	5.8
Peptide N	2.3	2.4	2.7	1.7	2.2	3.0	2.9
U.S.O. N ††	3.1	4.7	7.9	3.7	11.1	3.3	10.5
Nitrate N	10.1	8.6	7.5	8.6	5.8	5.4	4.5
Ammonium N	0.2	0.0	0.2	0.4	0.2	0.9	1.6
				Roots			
Total N	38.4	40.3	39.0	29.8	41.5	28.9	43.3
Organic N	31.6	37.8	35.6	28.2	39.6	28.0	42.2
Protein N	25.9	31.0	28.5	21.8	31.6	22.8	33.4
Sol. org. N	5.7	6.8	7.1	6.4	8.0	5.2	8.8
Amino N	2.1	1.7	1.8	1.2	1.8	1.2	2.1
Amide N	1.6	0.8	0.9	0.4	0.3	0.2	0.4
Peptide N	1.8	1.3	1.2	1.5	1.5	1.6	1.5
U.S.O. N ††	0.2	2.9	3.2	3.3	4.4	2.3	4.8
Nitrate N	6.8	2.5	3.2	1.2	1.6	0.4	0.8
Ammonium N	0.0	0.0	0.1	0.4	0.3	0.4	0.3

* Expressed as mg N/plant part.
** Plants treated one day with 5 ppm 2,4-D in nutrient solution.
† No treatment with 2,4-D.
†† Undetermined soluble organic N by difference.

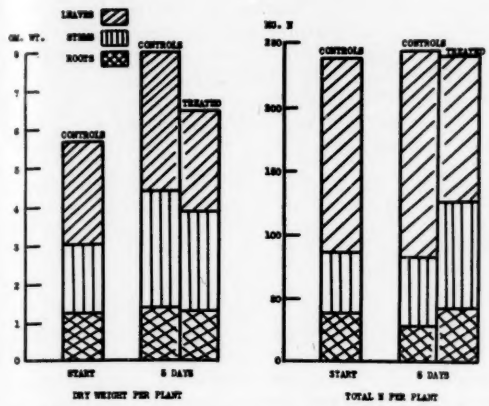

FIG. 1. Total dry wt and total N/plant in leaves, stems, and roots at start of treatment and 5 days after treatment with 2,4-D.

2,4-D introduction were smaller than in the previous harvests of treated plants and far smaller than in the control leaves of any of the four harvests.

Despite the differences between treated and control plants with respect to N fractions or total N in a given organ, total N in the whole plant was approximately the same in treated plants and controls at any one time (fig 1) and constant for the 5-day period of the experiment. Such differences in total N per plant as did occur were apparently related to differences in size of plants harvested in the several groups. These data also indicate that neither treated nor control plants lost nitrogen to an extent that could be detected by analyses of replicate lots of plants.

Because no source of external nitrogen was available to treated or to control plants after the start of exposure to 2,4-D, and because the total N content of both groups remained approximately the same, any changes in the N content of the separated organs were the result of translocation to or from a given organ. Without question, 2,4-D resulted in a translocation of N from the leaves mainly to the stems but also to the roots. This is brought out by figure 1, which shows the magnitude of this translocation which occurred in spite of the fact that the treated plants did not increase in size or in weight of leaves, stems, or roots to the extent that the controls did.

Five days from the start of treatment the protein N content in the leaves of treated plants had decreased approximately 36 mg, which amounted to a 30 % loss after the start of exposure to 2,4-D. During the same interval the protein N content in the stems of the treated plants increased by 26 mg, amounting to a 104 % gain, and in the roots protein N increased 7 mg for a 27 % gain. These estimates, although admittedly affected slightly by individual plant size, indicate the marked influence of 2,4-D in changing the location of a considerable amount of protein in all organs of the plant and particularly in the stems.

In all organs of the treated plants the concentration of soluble organic N was greater than in the corresponding control organs by the third day after 2,4-D introduction (table II). On a per plant basis also (table III), increases in soluble organic N in treated stems and roots above levels found in control stems and roots were evident, but because of increased total dry weight of control leaf tissue, smaller amounts of soluble organic N were found in treated leaves than in controls by the fifth day after 2,4-D introduction.

Increased soluble organic N in the stems and roots of treated plants was principally undetermined soluble organic N, as brought out in figure 2. This fraction differs from the residual soluble organic N fraction detailed in an earlier study (7) in that it does not include any peptide N, which was analytically determined in the present experiment.

By the fifth day the concentration as well as the total amount of undetermined soluble organic N in the stems and roots of treated plants rose to levels which were considerably above those found in the corresponding organs of the control plants harvested at that time or earlier. In some instances this fraction of undetermined forms of soluble organic N amounted to over 50 % of the soluble organic N in the treated stems and roots. Five days after treatment the total amount of undetermined soluble organic N in the stems was about 3 times that in the controls harvested at the same time.

The concentration of amino N in the controls decreased in all organs during the five days of growth in a minus-N solution. By the fifth day after treatment in all organs of the treated plants the concentration of amino N was higher than that in the corresponding controls of that date, and of the same

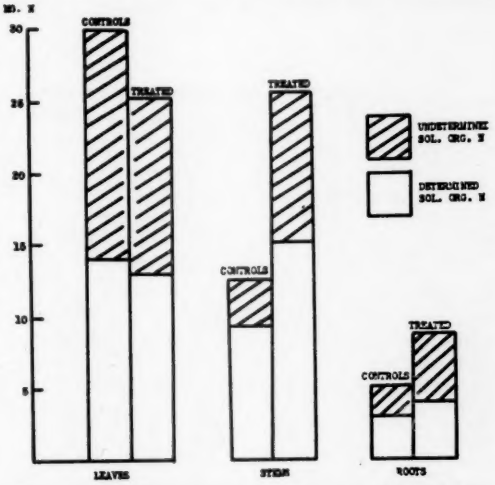

FIG. 2. Total amounts of soluble organic N/plant in undetermined and determined forms in leaves, stems, and roots of plants 5 days after treatment with 2,4-D.

order of magnitude as that in the plants harvested before treatment. On a per plant basis, amino N in the stems and roots was higher in the treated plants than in the corresponding controls, with a maximum difference reached by the fifth day.

Amide N showed a rapid decrease in concentration in control stems and roots after exposure to the minus-N nutrient solution. Treated plants showed somewhat higher concentrations than control plants by the fifth day, but only in the stems was the difference a striking one.

The concentration of peptide N was the most constant of all the N fractions determined, showing no real effect of growth of the plants in a minus-N solution or exposure to 2,4-D. Furthermore, the concentration was in the same range in leaf, stem, and root tissues.

In all organs of treated and control plants decreasing amounts of nitrate N were found with increasing duration of exposure of plants to nutrient solutions containing no nitrogen after introduction of 2,4-D. At any one harvest, the total amount of nitrate N in a given organ was approximately the same in treated and control plants. Treatment with the growth regulator, therefore, did not affect the assimilation of nitrate N in the plant.

Proteolytic Enzymes: The results shown in table IV indicate that the proteinase and peptidase activity of extracts from the control leaves, stems and roots remain fairly constant throughout the experiment. However, in extracts from the leaves of treated plants the proteinase was somewhat lower than in the controls after one day of treatment, and both the proteinase and peptidase activity markedly lower by the fifth day. In the extract from treated stems the peptidase activity remained similar to that of the controls until the fifth day after treatment when an increase was noted; however, the proteinase activity of the extracts of the stems was markedly higher than in the controls by the third day after treatment, and remained high throughout the experiment. In the extracts of roots of treated plants, a marked increase was noted in peptidase activity by the third day, and the increase in proteinase activity noted in the first day was accentuated in material from the third day.

No root tissue from the fifth day harvest was available for an analysis of proteolytic enzyme activity. Also, an insufficient quantity of leaf tissue was on hand from the first day harvest for analysis of peptidase activity. Consequently, results for those samples are lacking in table IV.

Discussion

The lack of an early significant effect of 2,4-D upon the percentage dry matter in leaves in this study, in contrast to the significantly lower percentage dry matter in treated than in control leaves found in an earlier report (7), is interpreted on the basis of differences in environmental factors affecting plants in the different experiments. In the present study, leaves of control plants harvested in the morning before 2,4-D treatment on a clear day with relative humidity of 52 to 78 % included 16.7 % dry matter, as contrasted with 14.5 % in controls harvested one day later on a cloudy day with 98 % relative humidity. Under conditions of high humidity the control leaves apparently lost little water. Under such conditions, any possible reduction of transpiration caused by the growth-regulator, with consequent reduction in percentage dry matter, could not have produced measurable differences from controls, which were also prevented from transpiring by the high humidity. Harvests of several species made on bright days have generally shown (7) an early decreased percentage dry matter following treatment of plants with 2,4-D, and this response has been interpreted as

Table IV

Proteinase and Peptidase Activity * of Soybean Plants Harvested at Intervals after Treatment with 2,4-D **

Plant parts	Days harvested after treatment						
	0	1		3		5	
	Cont †	Cont	2,4-D	Cont	2,4-D	Cont	2,4-D
			Proteinase				
Leaves	1.97	1.88	1.48	1.68	1.47	1.82	1.22
Stems	0.14	0.10	0.19	0.04	0.52	0.09	0.34
Roots	0.43	0.59	1.01	0.42	1.28	...	...
			Peptidase				
Leaves	2.55	...	...	2.49	2.35	2.62	1.55
Stems	0.77	0.84	0.90	0.66	0.79	0.75	1.10
Roots	0.86	1.16	1.25	0.81	1.65	...	...

* Activity measured as ml 0.05 *N* NaOH required to neutralize the increase in carboxyl groups after 24-hr incubation of plant extract with gelatin as substrate for proteinase measurement or peptone, for peptidase.

** Plants treated one day with 5 ppm 2,4-D in nutrient solution.

† No treatment with 2,4-D.

an effect of treatment upon the water relations of the plant.

Attention should be focused on the relationship of changes in protein content of the several organs of the plant to changes in activity of proteolytic enzymes and to changes in the distribution of soluble organic N fractions following treatment with 2,4-D. With plants lacking nodules on their roots when grown in solution culture, and deprived of a source of N in the culture solution after exposure to 2,4-D the general course of N metabolism in treated and control plants was fairly clear.

It appears to be unequivocally established by this experiment that there was a considerable hydrolysis of protein in the leaves of treated plants, and that the products of this hydrolysis moved to the stems, and perhaps to the roots, where a resynthesis of protein occurred, accompanied also by a definite accumulation of soluble organic N.

About 14 mg of nitrate N per plant were reduced by both treated and control plants during the five days from start of exposure to 2,4-D. The quantity of N thus added to the pool of organic N in the plant was not large in comparison with the amounts of N involved in changes in all organs as a result of treatment with 2,4-D.

The levels of soluble organic N fractions found in the different organs during the course of treatment probably have some bearing on changes in distribution of protein in the plant. Some increase in the total amount of amino N and amide N per plant was noted in the stems of treated plants where accumulation of protein was taking place, with lesser effects of treatment found in leaves and roots. Peptide N showed the least differences between controls and treated plants of all the soluble N fractions, forming a rather constant percentage of the dry weight of tissue at all stages. This meant, of course, that peptide in proportion to soluble organic N really decreased considerably in the stems and roots of treated plants because the level of soluble organic N increased in these organs considerably above that found in corresponding controls.

The very great increase in undetermined forms of soluble organic N, particularly in the stems, following treatment with 2,4-D, suggests that further attention should be given to this fraction. Possible components present as undetermined forms of N might be the non-amino N of arginine, histidine, proline, and tryptophan, or purine N, or the N of the simpler bases, alkaloids, or vitamins (14, 15).

There was a certain pattern of consistency in the effects of 2,4-D on proteolytic activity in soybeans in that proteinases and peptidases were affected similarly in a given tissue, showing decreased activity in the leaves but increased activity in the stems and roots following treatment with the growth regulator. The relationship of these changes in proteolytic activity to changes in protein levels in corresponding organs of treated plants is not clear. Higher proteolytic activity in extracts of stem tissues which formed more protein as a result of 2,4-D treatment appears to be paradoxical. So does the reduced proteolytic activity found in extracts of leaves of treated plants which decreased in total protein while control leaves were increasing.

The paradox is not considered to be related to the procedures employed. It is recognized that the drying technique used results in a considerable loss of proteolytic activity. The low concentration of proteolytic enzymes in green leaf tissue and retention of enzyme on the fiber (16) leads to serious analytical problems. Present techniques can be expected to give only relative values. Nevertheless, effects of 2,4-D on proteolytic activity in extracts of leaves and stems in our experiments are essentially the same as those of Rebstock et al (11) whose study differed from ours in species treated, technique of exposure to 2,4-D, method of drying the tissue, and technique of measuring proteolytic activity.

It appears, however, that these in vitro measurements of proteolytic activity in extracts of tissues are not a suitable index of the prevailing equilibrium between hydrolytic and synthetic reactions within the plant.

Perhaps the activity (concentration) of proteolytic enzymes found was related primarily to the level of protein and to the degree of meristematic activity in a given tissue. In the stems of treated plants, increased meristematic activity accompanying proliferation of root initials and greater accumulation of protein accompanied an increase in proteinase activity. At the same time, the roots of treated plants, which also showed greater meristematic activity and a higher protein content, were higher in proteolytic activity than control roots. These results suggest an elaboration of enzyme-protein in tissues synthesizing more total protein as a result of treatment with 2,4-D. In contrast, in the leaves where the level of protein decreased as a result of treatment with 2,4-D, a lower level of proteinase and peptidase activity was found.

Summary

Soybean plants grown in solution and treated with 2,4-D were analyzed for several nitrogen constituents and for proteolytic enzyme activity. Control and treated plants were both grown in a minus-N solution during and following a 25-hr exposure of the latter to 5 ppm 2,4-D added to the nutrient solution.

1. Essentially no change in total dry weight of treated plants occurred after exposure to 2,4-D.

2. It is suggested that the very high relative humidity prevailing at the time of the harvest was responsible for the lack of difference in percentage dry matter of leaves between treated and control plants.

3. Nitrate assimilation was equally rapid in treated and control plants.

4. The N distribution in the different plant organs was greatly affected by 2,4-D treatment, although no difference was found in total N content between treated and control plants as a whole.

5. By the third day after start of treatment, pro-

tein N on a per plant basis was lower in the leaves and much higher in the stems and roots of treated plants than in corresponding tissues of controls harvested at the same time or at the start of treatment. Apparently some of the protein in the leaves of treated plants was hydrolyzed and the products were translocated to the stems and roots where more protein was resynthesized.

6. Marked increases in soluble organic N were found in the stems and roots of treated plants above that found in controls harvested at the start of treatment. Most responsible for this increase was the fraction containing undetermined forms of soluble organic N.

7. Proteolytic activity in extracts of all organs of the plants was markedly affected by treatment with 2,4-D. Decreased proteinase and peptidase activity in leaves and increased proteinase and peptidase activity in stems and roots followed treatment with 2,4-D.

The authors wish to express appreciation to members of the Microbiology Department of the New Jersey Agricultural Experiment Station for the culturing of *Aspergillus wentii* used in determining peptide nitrogen.

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STUDIES ON AN ENZYME SYSTEM FROM WHEAT GERM CATALYZING THE AEROBIC OXIDATION OF REDUCED TRIPHOSPHOPYRIDINE NUCLEOTIDE[1,2]

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Conn et al (3) found in water extracts of wheat germ a soluble protein fraction which catalyzed the oxidation of reduced triphosphopyridine nucleotide (TPNH) by molecular oxygen. To the enzyme system exhibiting this oxidase activity they gave the name TPNH oxidase. Electrophoretically they were able to separate from the crude protein fraction two protein components, both of which were necessary for the full oxidase activity and one of which was a peroxidase. The peroxidase component could be replaced by crystalline horseradish peroxidase showing that it was peroxidase per se that was a necessary adjunct to the oxidase system. A wide variety of compounds were found to inhibit the oxidase activity, among them compounds known to inhibit heme and copper enzymes. However, using the succinic oxidase system of pig kidney as an assay system, they were able to exclude cytochrome c as a component of the

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TPNH oxidase. The number of enzymes in this newly found pathway to oxygen and the prosthetic groups of these enzymes are not known, nor has the role of peroxidase in this system been elucidated.

The results of the experiments presented in this paper demonstrate the occurrence of a TPNH oxidase in wheat germ "malic" enzyme preparations which is similar to or perhaps identical with the oxidase of Conn et al (3). This crude "malic" enzyme preparation catalyzes the following reactions.

(1) malate + TPN^+ ⟶ pyruvate + CO_2 + TPNH + H^+
(2) TPNH + H^+ + ½ O_2 ⟶ TPN^+ + H_2O

(3) malate + ½ O_2 ⟶ pyruvate + CO_2 + H_2O

Evidence is presented for a cofactor and manganous ion requirement for full activity of the oxidase system catalyzing reaction (2). The cofactor, as yet unidentified, has been found in glutathione isolated from yeast and in crude coenzyme A preparations from liver.

Materials and Methods

Triphosphopyridine nucleotide of 10 % purity was prepared from sheep liver by an unpublished method of Kornberg and Horecker. Muscle adenylic acid and glucose-6-phosphate were purchased from Schwarz Laboratories, Inc. Coenzyme A of 0.9 % purity (prepared from hog liver) was purchased from the A. S. Aloe Company. Coenzyme A of 75 % purity (prepared from brewer's yeast) was purchased from Pabst Laboratories. Glucose-6-phosphate dehydrogenase was prepared from brewer's yeast by the method of Kornberg (13). Glutathione was purchased from Schwarz Laboratories, Inc. and from General Biochemicals, Inc. or was prepared from baker's yeast by the method of Pirie (17). Oxidized glutathione was purchased from Schwarz Laboratories, Inc. or was prepared from glutathione by oxidation with H_2O_2 according to the method of Pirie (18), except that no copper was used as a catalyst. L-malic acid, C.P. grade, was purchased from Pfanstiehl Chemical Co. and was used without further recrystallization. Sodium thioglycolate, practical grade, was purchased from Eastman Kodak Co. All other chemicals used in these experiments were of reagent grade or of the highest purity commercially available.

The protein preparations employed in these experiments were made from Gold Medal wheat germ (General Mills, Inc.), and from wheat germ "B" (B. A. Eckhart Milling Company). Both brands of wheat germ were specified to be "not heat treated." The protein solutions were prepared by ammonium sulfate fractionation of water extracts of wheat germ according to the type C wheat germ procedure of Conn et al (4) for the preparation of the "malic" enzyme, except that the protein precipitates were dissolved in and dialyzed against phosphate buffer of pH 6.8 instead of pH 7.3. Protein solutions obtained by this procedure will be referred to as preparation I. They contained from 85 to 90 mg of protein per ml. In some instances the protein that precipitated at an $(NH_4)_2SO_4$ concentration of 290 gm/l of protein solution (step 3 of the procedure of Conn et al (4) for type C wheat germ) was used. These protein solutions will be referred to as preparation II. They contained from 87 to 98 mg of protein per ml. The protein solutions were kept for as long as four months in the deep freeze with no noticeable loss of enzyme activity.

Muscle adenylic acid was added to the TPN solutions used in these experiments to prevent the hydrolytic cleavage of the TPN molecule by enzymes present in wheat germ protein preparations (4). The TPN-adenylic acid solutions were made to contain 100 μg of TPN and 5 mg of muscle adenylic acid per ml.

Pyruvic acid determinations were made by the specific method of Friedemann and Haugen (5). Glutathione (GSH) was estimated by titration with 0.001 *N* potassium acid iodate according to the method of Fujita and Numata (6).

The manometric experiments were conducted in the Warburg apparatus at 25° C. The reaction volume, in all cases, was 2.0 ml. For the measurement of oxygen uptake the vessels were filled with air and 0.2 ml of 10 % KOH was added to the center well. The "direct method" for the measurement of carbon dioxide evolution (19) was used.

Experimental Results

Cofactor Requirement: The presence of an oxidase system in crude "malic" enzyme preparations from wheat germ became evident when rather high rates of oxygen uptake were obtained with the system: crude "malic" enzyme, malate, TPN, $MnCl_2$, oxidized glutathione (GSSG). The oxidase was neither ascorbic acid oxidase nor cytochrome oxidase since no oxygen uptakes were obtained when ascorbic acid or hydroquinone and cytochrome c were added to the "malic" enzyme protein. It is very unlikely that cytochrome oxidase would be present since the "malic" enzyme protein was prepared from a simple water extract of wheat germ, and Goddard (7) has found that wheat germ cytochrome oxidase is not water soluble unless the germ is first treated with acetone. Polyphenol oxidase is apparently eliminated also since this enzyme has not been found in wheat germ (7). Following Conn et al (3) this enzyme system catalyzing the aerobic oxidation of TPNH will be referred to as TPNH-oxidase.

That the system has an apparent requirement for GSSG is shown in figure 1 where the results of a typical experiment are shown. The initial 10 minute lag period in the glutathione curve is most likely due to the action of glutathione reductase which is present in the wheat germ protein fraction along with the "malic" enzyme and TPNH oxidase. Glutathione reductase catalyzes the reaction

(4) TPNH + H^+ + GSSG ⟶ TPN^+ + 2 GSH

and would compete with the TPNH oxidase for reduced TPN. It was found, by measuring the appearance of reduced glutathione (GSH) that reaction 4

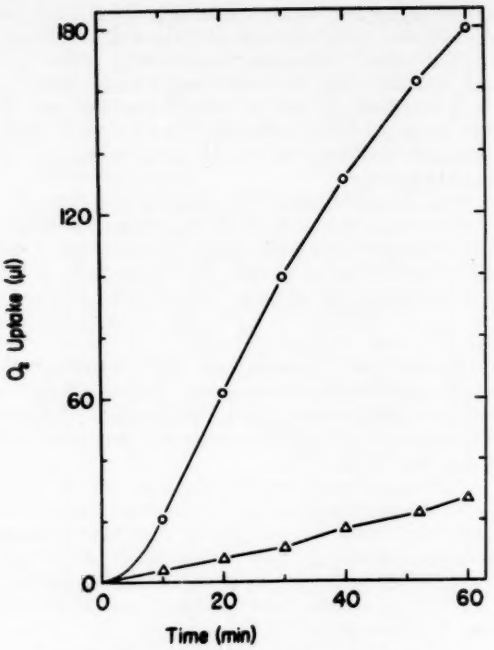

FIG. 1. Effect of GSSG on O_2 uptake of the "malic" enzyme-TPNH oxidase system. *Complete system.* 0.2 ml malate (0.83 M); 0.2 ml TPN-muscle adenylic acid solution (20 µg TPN, 1 mg adenylic acid); 0.05 ml $MnCl_2$ (0.128 M); 0.5 ml preparation I wheat germ protein solution (42 mg protein); 0.05 ml phosphate buffer (0.2 M, pH 7.4); 0.2 ml GSSG solution (2 mg GSSG); H_2O to 2.0 ml. The reaction was started by tipping in the TPN-adenylic acid solution at zero time. All solutions were adjusted to pH 6.6 to 6.8 before use. Initial pH 6.6, temperature 25° C. ○○○—Complete. △△△—Complete minus GSSG.

goes to completion in 12 to 14 minutes. The effect of added GSSG could not be due to the autoxidation of the GSH formed since added GSH had no effect on the oxygen uptake. Cysteine and cystine likewise had no effect on the oxygen uptake.

Numerous commercial samples of GSSG were tested for this stimulatory action. Most of the samples tested had no effect, two samples gave a slight increase, and only the original sample (one gm Schwarz GSSG) used gave a large increase (of the magnitude shown in fig 1) in oxygen uptake. Numerous experiments were run using the original sample of GSSG and the results were quite reproducible. Marked increases in O_2 uptake (similar to those shown in fig 1) were obtained with preparations of glutathione isolated from baker's yeast and oxidized with hydrogen peroxide (17, 18), but these preparations were active for only 3 to 4 days after oxidation. In no case was GSH found to be active. The activity became evident only after oxidizing GSH to GSSG. From these results it was concluded that some impurity in the glutathione was responsible for the stimulation of O_2 uptake. Further, since GSH was found to be inactive, it was concluded that either the action of the impurity is inhibited by GSH or the impurity is only active in the oxidized state.

Because coenzyme A (CoA) has been shown to be an impurity in commercial glutathione preparations (20), a crude (0.9 % CoA) liver CoA preparation was tested for cofactor activity. CoA solutions were prepared to contain 112.5 µg CoA per ml (12.5 mg of the crude liver powder per ml). Some of the solutions were made 1.3×10^{-2} M with respect to hydrogen peroxide and placed in the cold for at least 40 hours before use. The results of a typical experiment are plotted in figure 2. In the experiments run, the sharp increase in the untreated CoA curve always occurred between 70 and 80 minutes after tipping in the malate. The hydrogen peroxide treated CoA solutions contained an excess of hydrogen peroxide which caused a marked depression in the O_2 uptake, since hydrogen peroxide inhibits the action of the "malic" enzyme (8). Because the crude wheat germ protein preparation used in these experiments had catalase activity, the excess hydrogen peroxide could be removed by shaking the Warburg vessels (open to the air) for at least 15 minutes before tipping in the malate.

Purer CoA preparations (75 % pure) had no stimulatory effect on the system, even at CoA levels triple those used in the crude CoA experiments. Both

TABLE I

FORMATION OF CO_2 BY THE "MALIC" ENZYME-TPNH OXIDASE SYSTEM

EXPERIMENT NO.	SYSTEM *	O_2 UPTAKE	CO_2 EVOLVED	CO_2/O_2
		µl	µl	
1	Complete minus cofactor	55	100	1.82
2	" " "	55	92	1.70
3	Complete (2 mg GSSG)	208	365 **	1.75
4	" "	220	338 **	1.54
5	Complete (10 mg untreated crude CoA)	304	429	1.41
6	Same as experiment 5	310	449	1.45
7	Complete (10 mg H_2O_2 treated crude CoA)	316	503	1.59

* Complete system: 0.2 ml malate (0.83 M); 0.2 ml TPN-muscle adenylic acid solution (20 µg TPN, 1 mg adenylic acid); 0.1 ml $MnCl_2$ (0.0128 M); 0.5 ml preparation I wheat perm protein solution (42 to 45 mg protein); cofactor source as indicated in table; H_2O to 2 ml. The reaction was started by tipping in the malate. All solutions were adjusted to pH 6.6 to 6.8 before use.

** Values corrected for the effect of GSSG. The coupling of reactions 1 and 4 produces CO_2 and pyruvic acid but no O_2 uptake results. Therefore 40 µl for each mg of GSSG used were subtracted from the CO_2 value obtained in order to arrive at a meaningful CO_2/O_2 ratio. The GSSG used was found to be 60 % pure by measuring the GSH formed after complete reduction of the GSSG with glutathione reductase. This purity value was used in calculating the corrections.

TABLE II

FORMATION OF PYRUVIC ACID BY THE "MALIC" ENZYME-TPNH OXIDASE SYSTEM

EXPERIMENT NO.	SYSTEM *	O_2 UPTAKE	PYRUVIC ACID	
			FOUND	CALCULATED **
		μl	*mg*	*mg*
8	Complete minus cofactor	33.5	0.24	0.26
9	" " "	35.8	0.25	0.28
10	Complete (2 mg GSSG)	161	1.47	1.44 †
11	" "	179	1.55	1.59 †
12	Complete (7.5 mg H_2O_2 treated crude CoA)	253	1.95	1.99
13	Same as experiment 12	195	1.51	1.53
14	Complete (7.5 mg untreated crude CoA)	73	0.54	0.57
15	Same as experiment 14	276	2.06	2.17

* Complete system: 0.2 ml malate (0.83 M); 0.2 ml TPN-muscle adenylic acid solution (20 μg TPN, 1 mg adenylic acid); 0.1 ml $MnCl_2$ (0.0128 M); 0.5 ml preparation I wheat germ protein solution (42 to 45 mg protein); cofactor source as indicated in table; H_2O to 2 ml. The reaction was started by tipping in the malate. All solutions were adjusted to pH 6.6 to 6.8 before use.

** Pyruvic acid was calculated from the O_2 uptake assuming 1 mole of pyruvic acid per 0.5 mole of O_2 (reaction 3).

† Value corrected for the effect of GSSG. (See footnote, table I.)

untreated and hydrogen peroxide treated solutions of the purified CoA were tested. Coenzyme A, then, is not the activating factor in this system. It seems unlikely that the activator (cofactor) is a sulfhydryl compound since the crude CoA solutions gave a negative nitroprusside test. Positive nitroprusside tests were obtained only after the addition of cyanide. The cofactor, however, may be present in concentrations too low to be detected by the nitroprusside test. The cofactor present in the crude CoA is not heat stable since the crude CoA (untreated) showed no activity after being heated to 100° C for 7 minutes.

The puruvate and the carbon dioxide formed by the system (reaction 3) were measured with the results shown in tables I and II. If equations 1 and 2 hold for the system under study, the CO_2/O_2 ratio and the ratio moles pyruvate/moles O_2 should both equal two. From these tables it can be seen that very good agreement with this value was obtained for the pyruvate/O_2 ratios. The CO_2/O_2 ratios varied, but were consistently well below two. The CO_2 and the pyruvate determinations were made for a range of O_2 uptake levels. In experiment 14 (complete; untreated CoA) pyruvate was measured just before the sharp increase in O_2 uptake occurred (see fig 2). In experiment 15 pyruvate was determined in the same type of system well after this sharp increase. In both experiments the pyruvate measured was in good agreement with the pyruvate calculated on the basis of one mole of pyruvate formed per 0.5 mole of O_2 taken up. This would hardly be the case if the observed O_2 uptake were the result of a bacterial contaminant.

The cofactor, from both glutathione and crude CoA, appears to be active only in the oxidized form. If this be true, then the behavior of the system to which untreated CoA (i.e., reduced cofactor) has been added (fig 2) can be explained by assuming that, instead of reaction 2, reaction 5 is occurring.

$$(5) \qquad TPNH + H^+ + O_2 \longrightarrow TPN^+ + H_2O_2$$

The hydrogen peroxide formed by reaction 5 would oxidize the reduced cofactor, and the time lag in the O_2 uptake curve obtained with this system (fig 2) would represent the time necessary to build up quantities of oxidized cofactor sufficient to support high rates of O_2 uptake. Because the wheat germ protein used in these experiments exhibits strong catalase activity hydrogen peroxide would not accumulate and reaction 2 would represent the overall reaction. The quantity of hydrogen peroxide necessary to oxidize the cofactor is so small that the effect on the pyruvate/O_2 ratio would be negligible.

To determine which reaction (reaction 1 or 2) required the cofactor, glucose-6-phosphate (G-6-P) and glucose-6-phosphate dehydrogenase were used instead of malate and the "malic" enzyme to reduce the TPN. Glucose-6-phosphate dehydrogenase catalyzes reaction 6.

$$(6) \quad G\text{-}6\text{-}P + TPN^+ + H_2O \longrightarrow 6\text{-phosphogluconate} + TPNH + H^+$$

The system: TPNH oxidase ("malic" enzyme protein), glucose-6-phosphate dehydrogenase, G-6-P, TPN, $MnCl_2$ (reactions 6 and 2), exhibited only low rates of O_2 uptake. The addition of crude, hydrogen peroxide treated CoA increased the O_2 uptake greatly. These results are plotted in figure 3. Since it is very unlikely that the cofactor would be required by both the "malic" enzyme and glucose-6-phosphate dehydrogenase, it must be concluded from these results that the cofactor acts somewhere along the pathway from reduced TPN to oxygen (reaction 2). That this conclusion is correct is further evidenced by the fact that enough glucose-6-phosphate dehydrogenase was added to each Warburg vessel to support an O_2 uptake of approximately 270 μl/ hr. The glucose-6-phosphate dehydrogenase was assayed spectrophotometrically by following the increase in optical density at 340 mμ due to the reduction of TPN. The assay system contained G-6-P, glucose-6-phosphate dehydrogenase, $MgCl_2$, buffer, and TPN; it did not contain the cofactor.

By using G-6-P and glucose-6-phosphate dehydrogenase to reduce the TPN it became possible to determine whether or not the oxidation of TPNH is enzymatic in this system, for the possibility remained that the wheat germ protein served merely as a catalyst ("malic" enzyme) in the reduction of TPN. From the experimental results shown in table III it can be seen that the wheat germ protein contains enzymes catalyzing the oxidation of TPNH. The decrease in activity of the complete system in later experiments

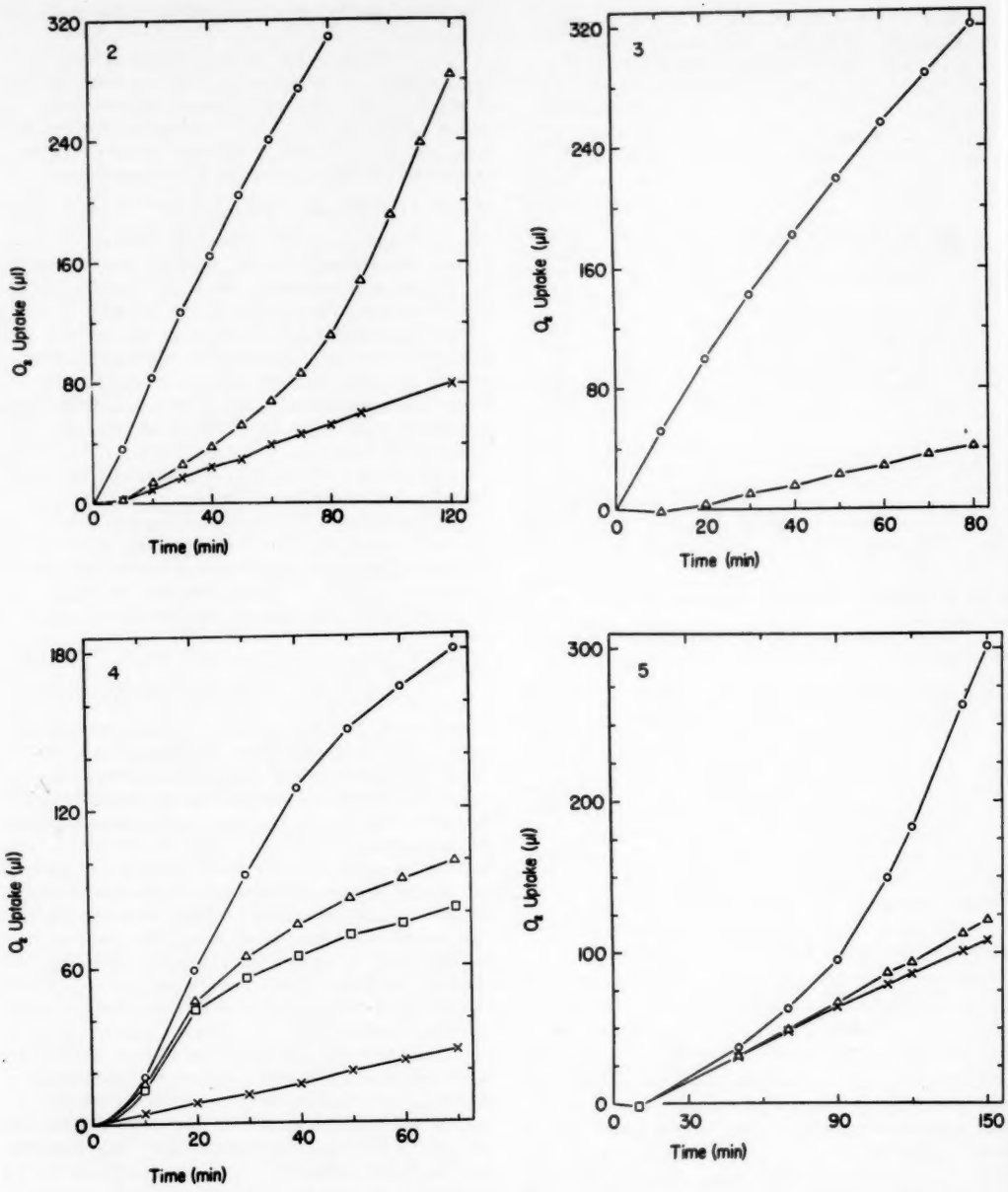

FIG. 2. Effect of crude liver CoA preparations on O_2 uptake. *Complete system.* 0.2 ml malate (0.83 M); 0.2 ml TPN-muscle adenylic acid solution (20 µg TPN, 1 mg adenylic acid); 0.1 ml $MnCl_2$ (0.0128 M); 0.5 ml preparation I wheat germ protein solution (45 mg protein); 0.6 ml of either untreated or H_2O_2 treated crude CoA (7.5 mg crude CoA); H_2O to 2.0 ml. The reaction was started by tipping in the malate at zero time. All solutions were adjusted to pH 6.6 to 6.8 before use. Initial pH 6.6, temperature 25° C. ○○○—Complete (7.5 mg H_2O_2 treated, crude CoA). △△△—Complete (7.5 mg untreated, crude CoA). +++—Complete minus crude CoA.

FIG. 3. Effect of cofactor on the glucose-6-phosphate dehydrogenase-TPNH oxidase system. *Complete system.* 0.2 ml G-6-P (0.25 M); 0.2 ml TPN-muscle adenylic acid solution (20 µg TPN, 1 mg adenylic acid); 0.1 ml $MnCl_2$ (0.0128 M); 0.1 ml glucose-6-phosphate dehydrogenase (0.4 units of activity); 0.5 ml preparation I wheat germ protein solution (45 mg protein); 0.6 ml of H_2O_2 treated crude CoA (7.5 mg crude CoA); H_2O to 2.0 ml. The reac-

TABLE III

REQUIREMENT OF WHEAT GERM PROTEIN FOR THE OXIDATION OF TPNH

EXPERIMENT NO.	SYSTEM *	O_2 UPTAKE † 60 MIN
		μl
1	Complete	257.0
2	Minus wheat germ protein	−3.6
3	Boiled wheat germ protein	−2.7
4	Complete	172.0
5	Complete	121.0
6	Minus wheat germ protein	−3.2
7 ††	Minus CoA and glucose-6-phosphate dehydrogenase	33.3
8 ††	Minus glucose-6-phosphate dehydrogenase; 2 mg GSSG instead of CoA	80.9

* Complete system: 0.2 ml G-6-P (0.25 M); 0.2 ml TPN-muscle adenylic acid solution (20 μg TPN, 1 mg adenylic acid); 0.1 ml $MnCl_2$ (0.0128 M); 0.1 ml glucose-6-phosphate dehydrogenase (0.4 units of activity **; 0.5 ml preparation I wheat germ protein solution (45 mg protein); 0.6 ml crude, H_2O_2 treated CoA (7.5 mg, 67.5 μg CoA); H_2O to 2 ml. The reaction was started by tipping in the G-6-P. All solutions were adjusted to pH 6.6 to 6.8 before use.

** A unit of glucose-6-phosphate dehydrogenase activity is defined (16) as the amount causing the reduction of 1 μM of TPN/min.

† Positive values refer to a gas uptake; negative values to a gas evolution.

†† 50 mg wheat germ protein was used in these experiments.

TABLE IV

Mn^{++} REQUIREMENT FOR THE OXIDATION OF TPNH

EXPERIMENT NO.	DIVALENT METAL AS CHLORIDE	METAL CONC M	O_2 UPTAKE 70 MIN	WHEAT GERM PREP
			μl	
	Complete * *(G-6-P dehydrogenase)*			
1	Mn	6.4×10^{-4}	202	I
2	Co	"	55	I
3	Mg	"	57	I
4	None	.....	57	I
	Complete * *("malic" enzyme)*			
5	Mn	3.2×10^{-4}	179	II
6	Mg	"	36	II
7	Mg	1.9×10^{-3}	46	II
8	Mg	3.2×10^{-3}	47	II
9	Co	3.2×10^{-4}	50	II
10	Co	1.9×10^{-3}	51	II
11	Mn	3.2×10^{-3}	134	I
12	None	.....	44	I
13	Mn	3.2×10^{-3}	262	I

* Two complete systems were used, the "malic" enzyme system and the glucose-6-phosphate dehydrogenase system according to the method employed to reduce TPN. Both systems contained: 0.2 ml TPN-muscle adenylic acid solution (20 μg TPN, 1 mg adenylic acid); 0.5 ml wheat germ protein solution (45 mg protein). The glucose-6-phosphate dehydrogenase system contained in addition 0.2 ml G-6-P (0.25 M); 0.1 ml glucose-6-phosphate dehydrogenase (0.4 units of activity); 0.6 ml crude, H_2O_2 treated CoA (7.5 mg, 67.5 μg CoA). The "malic" enzyme system contained in addition 0.2 ml malate (0.83 M); 0.2 ml GSSG (2 mg GSSG). The final volume of both systems was 2.0 ml.

(experiments 4 and 5) is probably due to the partial denaturation of the glucose-6-phosphate dehydrogenase caused by the freezing and thawing of the enzyme solution which was stored in the frozen state. The wheat germ protein used in these experiments has some glucose-6-phosphate dehydrogenase activity as is shown by the results of the experiments 7 and 8. Anderson et al (1) previously have demonstrated the presence of glucose-6-phosphate dehydrogenase in wheat germ preparations by coupling reactions 6 and 4 and measuring the GSH formed.

MANGANESE REQUIREMENT: Manganese chloride was routinely added to the Warburg vessels in these experiments because the "malic" enzyme is inactive in its absence. This requirement for Mn^{++} has been shown for highly purified pigeon liver "malic" enzyme preparations (16), and for crude "malic" enzyme preparations from higher plants including wheat germ (4). Though Co^{++} and Mg^{++} can substitute for Mn^{++}, Mg^{++} is much less effective. At an Mn^{++} concentration of 5×10^{-5} M the enzyme is half saturated; the Mg^{++} concentration for half saturation is 5×10^{-4} M (15). Kornberg (13) has obtained, in the presence of glycylglycine buffer, 3- to 4-fold increases in the activity of glucose-6-phosphate dehydrogenase (crude as well as purified preparations) by the addition of Mg^{++}. With phosphate buffer, however, the effect of added Mg^{++} was slight, and the activity of the glucose-6-phosphate dehydrogenase approached that obtained when glycylglycine and Mg^{++} were present in the reaction mixture. Since glycylglycine forms complexes with Mg^{++} (13) it may be assumed that glucose-6-phosphate dehydrogenase has a metal requirement that is fulfilled, in the absence of strong chelating

tion was started by tipping in the G-6-P at zero time. All solutions were adjusted to pH 6.6 to 6.8 before use. Initial pH 6.6, temperature 25° C. ○○○—Complete (7.5 mg H_2O_2 treated crude CoA). △△△—Complete minus CoA.

FIG. 4. Effect of thioglycolate on the complete ("malic" enzyme) system. *Complete system.* Same as in figure 1. ○○○—Complete. △△△—Complete + 0.1 ml sodium thioglycolate (8×10^{-2} M). □□□—Complete + 0.2 ml sodium thioglycolate (8×10^{-2} M). +++—Complete minus GSSG or complete minus GSSG + 0.1 ml sodium thioglycolate (8×10^{-2} M).

FIG. 5. Effect of GSSG and GSH on the "malic" enzyme-TPNH oxidase system when untreated crude CoA is the cofactor source. *Complete system.* Same as in figure 2. ○○○—Complete (7.5 mg untreated CoA). △△△—Complete (7.5 mg untreated CoA) + 2 mg GSH. +++—Complete (7.5 mg untreated CoA) + 2 mg GSSG. □—All three curves coincide at this point.

agents, by traces of metal normally present in reaction mixtures.

Experiments were performed to test the effect of Mn^{++}, Co^{++} and Mg^{++} on the "malic" enzyme-TPNH oxidase system (reactions 1 and 2), and on the glucose-6-phosphate dehydrogenase-TPNH oxidase system (reactions 6 and 2). From the results of these experiments, presented in table IV, it is evident that Mn^{++} is a requirement for both systems and cannot be replaced by Co^{++} or Mg^{++} even when the latter two metals are present in much higher concentrations than that of the Mn^{++}. The O_2 uptake in the presence of Co^{++} or Mg^{++} was, in all cases, nearly the same as that obtained when no metal was added, Co^{++} and Mg^{++} apparently have no activating effect whatsoever on either system. The residual O_2 uptake obtained when no metal was added probably reflects the presence of traces of metal impurities in the reaction mixture.

Since the "malic" enzyme requirement for Mn^{++} can be replaced by Co^{++} and by Mg^{++}, and since, in the absence of a strong chelating agent, glucose-6-phosphate dehydrogenase requires no metal supplement it must be concluded that the absolute Mn^{++} requirement of the two systems under study is a reflection of the metal requirement of the TPNH oxidase.

It should be noted that in these experiments (table IV) crude, hydrogen peroxide treated CoA served as a cofactor source for the glucose-6-phosphate dehydrogenase system, and GSSG served as a cofactor source for the "malic" enzyme system. Mn^{++} is required regardless of which source furnishes the cofactor.

INHIBITORS: The results of some inhibition studies are shown in table V. Ascorbic acid apparently inhibits the TPNH oxidase since inhibition was obtained regardless of the method used to reduce TPN (reaction 1 or 6). High inhibition with ascorbic acid was obtained only in the presence of the cofactor. In the absence of the cofactor no inhibition or low inhibition was obtained. Since the cofactor appears to be active only in the oxidized state, ascorbic acid may be acting by reducing the oxidized cofactor. The inhibition observed with cyanide is most probably due to its action on the TPNH oxidase since Van Heyningen and Pirie (8) found that their "malic" enzyme preparations from cattle lens were not inhibited by cyanide. Conn et al (3) also obtained inhibition of their TPNH oxidase preparations with both ascorbic acid and cyanide. Cupric sulfate has been reported to be an inhibitor of both the "malic" enzyme (8) and TPNH oxidase (3). Diethyldithiocarbamate and potassium cyanide inhibit heme and copper enzymes, but they also form complexes with metals as does ethylenediamine.

No inhibitions were obtained with the following substances: sodium azide (10^{-3} M), hydroxylamine (2×10^{-3} M), catalase, carbon monoxide (95 % CO, 5 % O_2). The rates of O_2 uptake in the control vessels for the carbon monoxide experiments (gas phase: 95 % N_2, 5 % O_2) were about 35 % below the rates for those vessels in which air was the gas phase, indicating that the terminal oxidase in the TPNH oxidase system has a rather low affinity for O_2.

Sodium arsenate (10^{-2} M) caused a slight stimulation in O_2 uptake. Whether or not this means an arsenolysis of phosphate esters was occurring remains to be shown.

The effect of sodium thioglycolate on the O_2 uptake of the complete ("malic" enzyme) system in which GSSG served as a cofactor source is shown in figure 4. Sodium thioglycolate in the concentration used has little effect for the first 20 minutes of the reaction. After this initial 20-minute period, however, it causes a sharp decrease in O_2 uptake. When hydrogen peroxide treated crude CoA served as a cofactor source thioglycollate inhibited only when GSSG (which did not contain the cofactor impurity) was also present in the reaction mixture. In this latter case, although a pronounced thioglycolate inhibition was observed, the curves obtained were not like those shown in figure 4 but were more nearly straight lines. Thioglycolate did not inhibit those systems to which no cofactor had been added.

TABLE V

INHIBITORS OF THE "MALIC" ENZYME AND TPNH OXIDASE

INHIBITOR	CONC	SYSTEM *	% INHIBITION
			Prep. I
Ascorbic acid	1.5×10^{-3}	Complete ("malic" enz.) minus cofactor	0
" "	"	" " " "	27
" "	"	Complete ("malic" enz.) CoA	73
" "	"	Complete ("malic" enz.) GSSG	80
" "	"	Complete (G-6-P dehydrogenase) CoA	63
			Prep. II
Diethyldithiocarbamate	10^{-3}	Complete ("malic" enz.) GSSG	68
Potassium cyanide	10^{-3}	" " "	76
Ethylenediamine	5×10^{-3}	" " "	43
Copper sulfate	10^{-4}	" " "	37

* The complete systems were similar to those described in table III except that the above systems contained Mn^{++} (6.4×10^{-4} M). The enzyme listed in parentheses refers to the method employed to reduce TPN (reaction 1 or 6). CoA and GSSG refer to the source of the cofactor. For the KCN experiments 0.2 ml of 2 M KCN were added to the center well of the Warburg vessel in place of KOH.

An interesting type of inhibition was obtained upon the addition of GSSG or GSH to complete ("malic" enzyme) systems (reactions 1 and 2) in which untreated crude CoA served as a cofactor source. Results of a typical experiment are shown in figure 5. In the presence of GSSG or GSH the sharp increase in O_2 uptake which usually occurs between 70 and 80 minutes after tipping in the malate is abolished. It must be emphasized that the GSSG used in these experiments was a commercial sample which contained no cofactor impurity. If the cofactor had been present in the GSSG sample a plot of the results would have looked similar to the curve shown in figure 1. The behavior of cysteine and cystine in this system was quite similar to that obtained with GSSG and GSH. None of these four compounds, however, had any effect on the O_2 uptake of the complete ("malic" enzyme) system when hydrogen peroxide treated, crude CoA served as a source of the cofactor. Holding to the assumptions that the active form of the cofactor is the oxidized form, that hydrogen peroxide is produced in the system (reaction 5), and that this hydrogen peroxide oxidizes the cofactor, it would appear that GSSG, GSH, cysteine and cystine inhibit the untreated CoA system by preventing the oxidation of the cofactor. The action of those four compounds is not due apparently to -SH groups for, although the GSSG would be reduced to GSH by the glutathione reductase present, there is no evidence for the presence in the wheat germ protein used of a cystine reductase such as has been found in baker's yeast by Nickerson and Romano (14). Furthermore, cystine reductase requires reduced diphosphopyridine nucleotide (DPNH) for activity. It is evident from these results why it was not possible to demonstrate cofactor activity in preparations of GSH even though such preparations were active after oxidizing them with hydrogen peroxide.

Discussion

From the experimental results presented in this paper it is obvious that crude wheat germ "malic" enzyme preparations contain enzymes capable of catalyzing the aerobic oxidation of reduced TPN. Since this oxidation was not inhibited by azide or carbon monoxide, and since the presence of cytochrome oxidase could not be demonstrated, it appears unlikely that the pathway to oxygen includes the cytochrome-cytochrome oxidase system. Rather, because the TPNH oxidase has a low affinity for oxygen and because there is a definite possibility that hydrogen peroxide is an end product of the oxidation, it might be assumed that a flavin enzyme is the functional terminal oxidase in this system. The validity of such an assumption remains to be seen.

The question arises as to whether the TPNH oxidase described in this paper is identical to that described by Conn et al (3). There appear to be some very fundamental differences for, although they showed no requirement for a cofactor or for Mn^{++}, the preparations of Conn et al (3) catalyzed the oxidation of TPNH at rates (on a protein basis) very similar to those reported in this paper for systems containing both the cofactor and Mn^{++}. Their preparations were assayed spectrophotometrically, however, using TPNH (reduced with sodium hydrosulfite) as a substrate, and their experiments lasted no more than 15 minutes. It may not be, in this case, permissible to compare the activities of the two preparations on the basis of total protein content because the preparations of Conn et al probably contain a much higher percentage of TPNH oxidase protein. Other differences are pointed out by a comparison of the inhibitor studies with the two preparations. Sodium azide and hydroxylamine strongly inhibited the TPNH oxidase of Conn et al (3) while the TPNH oxidase preparations used in the experiments herein reported were not inhibited by these substances. It should be noted, however, that the system under discussion in this paper is much more complex than that studied by Conn et al. This must be taken into account when comparing them.

No attempt was made in this study to show a peroxidase requirement for full activity of the TPNH oxidase. The protein solutions used in these experiments, however, gave strongly positive tests for peroxidase both with the purpurogallin method (2) and with the guaiacol method (21). Although Conn et al (3) very convincingly demonstrated the necessity of peroxidase for full activity of their TPNH oxidase preparations, it is difficult to set forth, on the basis of our knowledge of peroxidase reactions, a possible role of peroxidase in this system. It is particularly so, since it appears, from the results presented in this paper, that the oxidation of one mole of TPNH requires only 0.5 mole of O_2. In this connection, the work of Kenten and Mann must be mentioned (9, 10). These investigators obtained plant root extracts which catalyzed the oxidation of Mn^{++} in the presence of hydrogen peroxide, and they showed that this catalysis was due to the presence of a peroxidase system. They suggest the following mechanism:

$$AH_2 + H_2O_2 \xrightarrow{\text{peroxidase}} A + 2\,H_2O$$
$$2\,H^+ + A + 2\,Mn^{++} \longrightarrow 2\,Mn^{+++} + AH_2$$
$$2\,Mn^{+++} + \text{metabolites} \longrightarrow 2\,Mn^{++} + \text{oxidized metabolites}$$

AH_2 in the Kenten and Mann experiments was *p*-cresol, and the results of their experiments indicate that it is an intermediate and not the final oxidation product of this compound which brings about the oxidation of Mn^{++} (9). In later papers, Kenten and Mann (11, 12) brought forth evidence that oxalate, oxaloacetate, ketomalonate and dihydroxytartrate could act as metabolites in the above scheme. If such a mechanism were operative in the TPNH oxidase system, AH_2 and metabolites would seemingly have to represent TPNH or some reduced carrier between TPNH and oxygen. The above mechanism would not fully account for the Mn^{++} requirement of the TPNH oxidase since, because catalase is present in the wheat germ protein, the removal of Mn^{++} would only reduce the

O_2 uptake by one half. To account for the Mn^{++} requirement shown by the results reported in this paper, the enzyme system producing the hydrogen peroxide necessary for the functioning of the above mechanism would also have to require Mn^{++}. By the same reasoning, the cofactor would be necessary for the hydrogen peroxide producing system and not for the Mn^{++} peroxidase system shown above.

Summary

1. Crude preparations of the "malic" enzyme from wheat germ catalyze the following reactions.

A. malate + TPN^+ $\longrightarrow$ pyruvate + CO_2 + TPNH + H^+

B. TPNH + H^+ + ½ O_2 $\longrightarrow$ TPN^+ + H_2O

2. Evidence is presented for a cofactor and Mn^{++} requirement for full activity of the oxidase system catalyzing reaction B.

3. The cofactor, as yet unidentified, has been found in yeast glutathione preparations and in crude, liver coenzyme A preparations. The cofactor, however, is neither glutathione nor coenzyme A.

4. Evidence is presented that the cofactor exists in both the oxidized and reduced form; the oxidized form being the active form.

5. The requirement of the oxidase system (TPNH oxidase) for Mn^{++} is quite specific. Neither Co^{++} or Mg^{++} can substitute for Mn^{++}.

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FAT METABOLISM IN HIGHER PLANTS. III. ENZYMIC OXIDATION OF GLYCEROL[1]

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Glycerol is an important component of naturally occurring lipids. It serves as the polyalcohol in neutral fats and as the backbone for a large group of phospholipids. In animal and bacterial tissues there is evidence that glycerol is first phosphorylated to α-glycerolphosphate which is then dehydrogenated to dihydroxyacetone phosphate (DHAP). At this level, DHAP enters the glycolytic system and is converted to pyruvic acid, which may then enter the metabolic pool of the tissue (1–4).

The present investigation, as a part of a continuing program in this laboratory concerning fat metabolism in higher plants (5–7), suggests that the metabolic pathway for glycerol in peanut cotyledons is similar to that found in animal tissue. In essence, it has been observed that a complete system for the oxidation of glycerol to CO_2 involves two enzymic components, namely, (1) a mitochondrial system which contains a glycerol kinase and a α-glycerolphosphate dehydrogenase as well as the complete tricarboxylic acid cycle system, and (2) a non-particulate system of soluble proteins responsible for the conversion of triose phosphates to pyruvic acid.

METHODS

PREPARATION OF EXTRACTS:[3] Peanuts (*Arachis hypogea* L., var. Virginia Jumbo) were purchased from Manning's, Inc., San Francisco, and were germinated in flats of moist peat moss. Since certain batches of peanuts were contaminated with *Aspergillus niger* and a Rhizopus species, routine dusting of the peanuts with "Semesan"[4] prior to planting completely inhibited the growth of these molds. When 5–8 days old, the plants were harvested, the cotyledons removed, washed, and any blemishes removed. The peanut cotyledons were rinsed with 0.01 % 2,3-dimercaptopropanol (BAL) to remove traces of "Semesan," then triturated in a Waring blender at 4° C for 15 seconds at full line voltage in 0.2 M "TRIS" (Tris-(hydroxymethyl)-aminomethane) buffer at pH 7.4 which was also 0.5 M with respect to sucrose. The volume of buffer used was twice the weight of cotyledons.

The homogenate was filtered through two layers of cheesecloth and this filtrate was centrifuged at 600 × g for five minutes at 4° C, the sediment was discarded and the supernatant subjected to a centrifugation at 10,000 × g for 30 minutes (high speed head, Refrigerated International Centrifuge #1). The supernatant contained the enzymes of the glycolytic cycle and is called the soluble protein fraction (SPF). The sediment was resuspended in the TRIS-sucrose buffer and recentrifuged at 10,000 × g for 30 minutes. This sediment was resuspended in the TRIS-sucrose buffer to a volume containing approximately 2 to 4 mg protein-nitrogen per ml. This particulate material is identified as a mitochondria preparation (MP) for two reasons: (a) the centrifugal force used for sedimentation is of the same order employed for the preparation of typical plant mitochondria (8), and (b) this material carries out the oxidative reactions of a TCA cycle with concomitant esterification of inorganic phosphate into high energy linkages of ATP. This property is considered to be a distinguishing biochemical characteristic of mitochondria.

REAGENTS: Adenosine triphosphate (ATP) was purchased from the Pabst Laboratories. Coenzyme 1 (DPN) and cytochrome c were obtained from the Sigma Company. Glycerol-1-C^{14} was prepared by the Research Specialties Company, Berkeley, California, and had a rated specific activity of 1 mc per millimole. The substrate was diluted with distilled water so that 0.1 ml was equivalent to 10^5 cpm and represented approximately 0.4 μmoles of glycerol. The product was radiochemically homogeneous as determined by one dimensional paper chromatography with either butanol-acetic-water or phenol-water as the developing solvent.

METHODS: The oxidation of glycerol-1-C^{14} was followed in conventional Warburg cups with 0.2 ml of 20 % KOH in the center well to trap $C^{14}O_2$ which is released by enzymic oxidative reactions. At the end of the reaction time, the alkali from the center well was transferred quantitatively to a conical centrifuge tube and precipitated as $BaCO_3$ by the addition of 0.2 ml of 20 % barium acetate. The precipitate was suspended in 50 % ethanol, centrifuged, and washed three times with 50 % ethanol, plated onto an aluminum disc and counted by a conventional Geiger-Muller counter.

Triose phosphate was estimated by the alkaline-hydrolyzable phosphate method (9). Inorganic phosphate was determined by the Fiske and Subbarow procedure (10).

To separate and isolate TCA cycle acids after oxidation of glycerol-1-C^{14} by the complete enzyme system, the reaction mixtures were deproteinized by the addition of two volumes of absolute alcohol. The deproteinized solution containing radioactive compounds was evaporated carefully to dryness and 0.5 ml of water added to redissolve the residue. This volume of solution was thoroughly mixed with 20 gm of fused $KHSO_4$ which was then packed into a glass column (150 mm × 10 mm). 50 ml of absolute ethyl

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[3] I wish to thank Dr. Mendel Mazelis for the preparation of the MP and SPF fractions.

[4] Semesan is a commercial fungicide containing hydroxymercurichlorophenol.

ether was passed through the column to elute the the ether-soluble compounds. The ether eluant was evaporated to dryness and redissolved in 0.1 ml of water. The solution was spotted on Whatman No. 1 paper and the spots developed in the long dimension with butanol-formic-water mixture (11). Radioautograms were made by placing the papers in contact with 14 × 17 inch Eastman medical "no-screen" x-ray film for two weeks. After this period of time the chromatograms were sprayed with 0.01 % bromcresol green to locate the acidic areas. In all cases the radioactive spots corresponded exactly with the acidic areas on the chromatogram and were tentatively identified by reference to spots of authentic TCA acids simultaneously run on the same chromatogram. The radioactive areas corresponding to the acidic spots were cut out, eluted, counted, and their identity confirmed by cochromatography with authentic samples of TCA cycle acids.

Results

When radioactive glycerol is added to a reaction mixture containing MP, SPF, and a number of cofactors and cosubstrates, rapid formation of radioactive CO_2 takes place. As summarized in table I the omission of any member of the complete system results in diminished or complete loss in activity. Thus removal of either MP or SPF leads to complete loss in activity. The removal of ATP, α-ketoglutaric acid, or cocarboxylase causes a serious impairment in oxidative activity. Without Mg^{++} and DPN^{+} there is an approximate decrease of 50 % and 65 % respectively. The presence of malonate and fluoride reduces oxidation to a low level.

These observations suggest that (a) the necessary enzymes for the oxidation process are found in the MP and SPF fractions, (b) ATP presumably serves as the source of ~ P for the phosphorylation of glycerol and for the maintenance of TCA cycle activity, (c) the requirement of a TCA cycle acid implies the participation of the TCA cycle in the formation of radioactive CO_2, (d) the inhibition of the formation of radioactive CO_2 by malonate is related to the known capacity of malonate to inhibit succinic dehydrogenase, and (e) the inhibition by fluoride suggests its involvement in the inhibition of enolase, the site of fluoride inhibition in the glycolytic cycle.

These results may be interpreted to mean that glycerol is first phosphorylated to α-glycerolphosphate which is then oxidized to DHAP. This intermediate enters the glycolytic system and is transformed to pyruvic acid which is then oxidized to CO_2 by the TCA system.

In support of this concept is (a) the evidence of an α-glycerolphosphate dehydrogenase in MP, (b) the accumulation of triose phosphate when α-glycerolphosphate is oxidized by MP, and (c) the labeling of members of the TCA cycle when glycerol-1-C^{14} is oxidized by the complete system.

α-Glycerolphosphate Dehydrogenase: The site of this activity is the mitochondrion. The soluble protein fraction is inert. α-Glycerolphosphate dehydrogenase activity can be followed by uptake of oxygen when α-glycerolphosphate is added to MP (table II), or by following the rate of reduction of redox dyes such as methylene blue, 2,6-dichlorophenolindophenol, or neotetrazolium. Fresh preparations of mitochondria are uniformly active whereas mitochondria that have been frozen, treated with acetone, lyophilized, or exposed to sonic vibration are inert. Exposure of MP to 55° C for 5 minutes completely inactivates the enzyme. The pH optimum is in the range of 6.8 to 7.3.

The stoichiometry of the reaction is established by the reaction:

α-glycerolphosphate + ½ O_2 → triose phosphate + H_2O

based on the data in table II. It is consistently ob-

Table I

Oxidation of Glycerol-1-C^{14} to $C^{14}O_2$ by Extracts of Peanut Cotyledons

Components	Total CPM as $BaC^{14}O_3$
Complete	12,500
without MP	0
without SPF	0
without ATP	56
without cytochrome c	13,100
without Mg^{++}	6,500
without α-ketoglutaric acid	600
without thiamine pyrophosphate	1,420
without DPN	8,500
Complete + malonate (10^{-1} M)	1,250
Complete + fluoride (10^{-2} M)	60

The complete reaction mixture contained 0.2 ml of MP (about 4 mg protein N per ml), 0.5 ml of SPF, 0.1 ml of glycerol-1-C^{14} containing 10^5 cpm and 0.4 μmoles of substrate, 0.1 ml of 0.01 M ATP, 0.1 ml of 10^{-6} M cytochrome c, 0.1 ml of 0.1 M $MgCl_2$, 0.1 ml of 0.1 M α-ketoglutaric acid, 0.1 ml of 0.1 % thiamine pyrophosphate, 0.1 ml of 0.1 % DPN, 0.5 ml of 0.2 M phosphate buffer at pH 7.2, 0.2 ml of 20 % KOH in center well, 0.3 ml of 10 *N* H_2SO_4 in side arm, and water to make a final volume of 3 ml. Incubated 2 hours, at 30° C.

Table II

Stoichiometry for Oxidation of α-Glycerol Phosphate to Triose Phosphate

Experiment	Oxygen uptake	Triose phosphate as alkaline-labile P
	μatoms	*μmoles*
1	10.1	8.8
2	5.2	4.8

Reaction mixture contained 0.2 ml of MP, 0.5 ml of 0.5 M DL-α,β-glycerol phosphate, 0.5 ml of 0.1 M TRIS buffer at pH 7.2, 0.2 ml of 20 % KOH in center well and water to final volume of 3 ml. Time 2 hrs, at 30° C. At end of incubation time oxygen uptake was measured and the reaction stopped by adding an equal volume of 10 % trichloroacetic acid to reaction mixture. Suspension was transferred to a test tube, diluted to 10 ml, filtered and aliquots analyzed for inorganic phosphate and alkaline labile phosphate.

served that for one atom of oxygen taken up, one molecule of triose phosphate accumulates. It is difficult to determine whether or not the first product of oxidation is DHAP or glyceraldehyde-3-phosphate since there is evidence that a triose-phosphate isomerase is present as a contaminant in the MP fraction. This enzyme catalyzes the conversion of triose phosphates to essentially DHAP.

No cofactor appears to be required by the preparation. The addition of either DPN or TPN had no effect on the rate of oxidation. Cytochrome c is very rapidly reduced as demonstrated by the results in figure 1. Since cytochrome c need not be added for

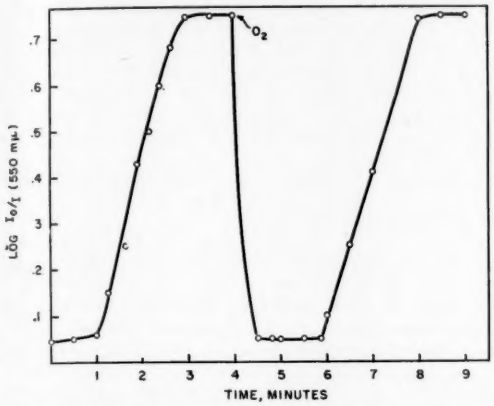


FIG. 1. The reduction of cytochrome c by the enzymic oxidation of α-glycerolphosphate as a function of time. The cuvette contained 0.1 ml of MP, 0.5 ml of 0.5 M DL-α,β-glycerolphosphate, 0.5 ml of 0.2 M phosphate buffer at pH 7.2, 0.5 ml of 10^{-4} M cytochrome c, and 1.5 ml of 0.2 M TRIS (pH 7.2) - 0.5 M sucrose buffer. Initially no increase in 550 mμ absorption (reduced cytochrome c) is noted until the dissolved oxygen in the system is exhausted by the oxidation of α-glycerolphosphate. After its removal a rapid formation of reduced cytochrome c is observed; reintroduction of oxygen immediately reoxidizes reduced cytochrome c since cytochrome oxidase is also present in the MP. The reaction mixture is then again reexamined for the 550 mμ band to ascertain the stability of the coupled oxidation reduction system.

maximum dehydrogenase activity it probably occurs in MP in a concentration which is not limiting. Presumably cytochrome c is the physiological redox acceptor for the dehydrogenase system. These results would indicate that plant α-glycerolphosphate dehydrogenase is similar to the particulate system found in animal tissue (12). The reaction sequence could be depicted as follows:

α-glycerolphosphate + cytochrome c
$\longrightarrow$ triose phosphate + reduced cytochrome c

reduced cytochrome c + $\frac{1}{2}$ O_2
$\xrightarrow[\text{oxidase}]{\text{cytochrome}}$ cytochrome c + H_2O

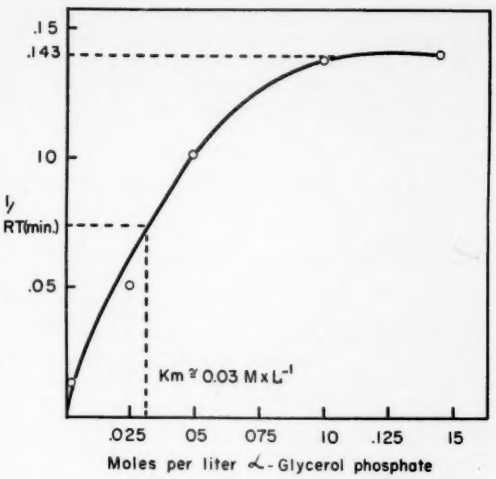


FIG. 2. The rate of methylene blue reduction as a function of α-glycerolphosphate concentration.

As indicated in figure 2, a rather high concentration of substrate is required to saturate the enzyme system. The approximate K_m is 0.03 M per liter. Because of the unavailability of L-α-glycerolphosphate, synthetic DL-α,β-glycerolphosphate was employed in this investigation. However, an authentic sample of L-α-glycerolphosphate was tested and found to be as active as an equivalent amount of the synthetic racemic mixture.

PARTICIPATION OF TCA CYCLE: If the TCA cycle is participating in the terminal oxidation of glycerol, then each member of the TCA cycle should become labeled when glycerol-1-C^{14} is oxidized by the complete system. In table III are summarized the results of an experiment in which glycerol-1-C^{14} was oxidized by the complete system for two hours in the presence of 10 μmoles of citrate as the sparking system. The data clearly indicate that the terminal oxidation of glycerol proceeds through the conventional TCA cycle localized in the mitochondria since appreciable radio-

TABLE III

INCORPORATION OF C^{14} INTO $C^{14}O$ AND TCA CYCLE ACIDS FROM GLYCEROL-1-C^{14}

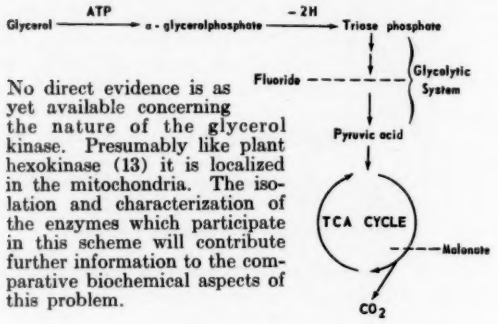
TCA CYCLE ACIDS	TOTAL CPM
Citric acid	1,950
Malic acid	3,230
α-Ketoglutaric acid	380
Succinic acid	704
Fumaric acid	1,100
(CO_2)	(12,000)

The reaction mixture contained 0.2 ml of MP, 0.5 ml of SPF, 0.1 ml of glycerol-1-C^{14} having 10^6 cpm, 0.1 ml of 0.01 M ATP, 0.1 ml of 0.1 M $MgCl_2$, 0.1 ml of 0.1% thiamine pyrophosphate, 0.1 ml of 0.1% DPN, 0.1 ml of 0.1 ml citrate, 0.2 ml of 20% KOH in center well, 0.5 ml of 0.2 M phosphate buffer at pH 7.2. Time 2 hours, at 30° C. See methods for isolation of TCA cycle acids.

activity is found in each of the members of the cycle. The variability of the radioactivity in each of the TCA cycle acids is probably related to the variability in recovery, the rates of formation and degradation in the mitochondria, and the size of the acid pools.

Discussion

The evidence presented in this paper may best be interpreted by the following scheme:



No direct evidence is as yet available concerning the nature of the glycerol kinase. Presumably like plant hexokinase (13) it is localized in the mitochondria. The isolation and characterization of the enzymes which participate in this scheme will contribute further information to the comparative biochemical aspects of this problem.

Summary

Glycerol is oxidized to CO_2 by the combined activities of (a) a mitochondrial preparation which contains presumably a glycerol kinase, glycerol phosphate dehydrogenase and the TCA cycle system, and (b) a soluble protein fraction which contains enzymes of the glycolytic cycle. The properties and cofactor requirements of this complete system are described.

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THE ENZYMATIC OXIDATION OF ASCORBIC ACID IN THE SLIME MOLD, PHYSARUM POLYCEPHALUM[1,2,3]

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The enzymatic oxidation of ascorbic acid was initially observed in cabbage leaves by Szent-Györgyi in 1930 (26). Since that time, numerous investigators have shown that this catalytic activity, which is present in a variety of plant juices, is due to the direct action of a specific enzyme, ascorbic acid oxidase, a copper protein (see reviews 7, 8, 15, and 22). Consequently, this activity is inhibited when the enzyme preparation is treated with substances which form stable complexes with copper; such substances are cyanide, sulfide, diethyldithiocarbamate (DDC), 8-hydroxyquinoline, ferricyanide, and carbon monoxide. Mandels (20, 21) has recently described an ascorbic acid oxidase from the fungus, *Myrothecium verrucaria*, which is insensitive to cyanide, azide, DDC, 8-hydroxyquinoline, etc., and apparently is not a copper oxidase.

An enzyme is present in homogenates of *Physarum polycephalum* which actively oxidizes ascorbic acid. This enzyme, which is not associated with a particu-

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[2] This represents part of a dissertation in Botany presented to the faculty of the Graduate School in partial fulfillment of the requirements for the degree of Doctor of Philosophy. This work was completed during the tenure of a National Institutes of Health Predoctoral Fellowship.

[3] This work was supported by a grant from the National Cancer Institute of the Public Health Service to Dr. David R. Goddard.

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late fraction, is insensitive to the respiratory inhibitors, cyanide, azide, sulfide, carbon monoxide in the presence and absence of light, and such copper complexing agents as DDC, 8-hydroxyquinoline, and ethylenediamine tetra acetic acid (Sequestrene). The unprecedented stimulation of this oxidation of ascorbic acid by DDC and tetraethylthiuram disulfide (TTD),[5] and the indirect evidence for the formation of H_2O_2 as an end product of this reaction serve to characterize this enzyme. It is the purpose of this paper to describe the unique properties of such an enzyme and to present evidence for the role of DDC and TTD in their stimulation.

Materials and Methods

Cultures of Physarum were grown on rolled oats according to the method of Camp (6). As the plasmodium migrates over the surface of the water it can be harvested relatively free of bacteria and protozoa. A plasmodium migrating on water is covered externally by a colorless, structureless, slimy material called a sheath which contains starch particles, bacteria, protozoa, and waste products and is left behind as the plasmodia advances (4). The presence of this sheath may be used to detect plasmodia which have transversed the same area more than once; such an area of the culture was not used for the harvesting of material. This procedure gave access to the most active portions of plasmodia and kept the ratio of sheath to plasmodium at a minimum, and the ratio of contaminant to plasmodium was also low.

Unless otherwise specified, the homogenates and extracts were prepared as follows. All operations were performed at 4° C, except the high speed centrifugation where the temperature was maintained at 1° C. Solutions, centrifuge tubes, mortar and pestle were pre-cooled before using. The mortar was charged with 10 gm wet weight plasmodia, 10 ml of 0.05 M, pH 7.2, KH_2PO_4-Na_2HPO_4 buffer and 5 gm of purified sand. Baker's technical sand ignited for boats was further purified by soaking in 6 *N* HCl at 100° C for 30 min, washed free of chloride ion, and dried in the oven. Homogenization was accomplished after 2 min grinding with a pestle, and the contents of the mortar were washed into the centrifuge tubes with an additional 2 ml of buffer. After centrifuging at 500 × g for 15 min, the sediment, which contained sand particles and large pieces of plasmodia, was discarded. The supernatant was yellow and opaque and was subjected to further centrifugation at 20,000 × g for 45 min, and the sediment was discarded. In this instance, the supernatant was yellow and clear and contained the enzyme.

Protein nitrogen was determined for each enzyme preparation by precipitation with trichloracetic acid, overnight digestion with H_2SO_4 in the sand bath at 240° C, and the addition of Nessler's reagent to the clear and colorless solution (11). The nitrogen content was then determined in a Klett-Summerson colorimeter using a filter with a transmission peak at 420 mμ.

Manometric experiments were performed using the Warburg constant volume respirometer at 25° C with a reaction mixture of 2.0 ml. The manometer fluid used was that described by Krebs (17). In experiments with cyanide, 0.02 ml 2 M KCN were placed in the center well in place of KOH to maintain a constant concentration of cyanide in the reaction mixture (18).

Results

The addition of 12 micromoles of neutralized ascorbic acid to homogenates of *Physarum polycephalum* results in an oxygen uptake curve (fig 1) with a rate of about 70 μl O_2 mg PN^{-1} hr^{-1}. An endogenous rate for the supernatant is of the order of 9 μl O_2 mg PN^{-1} hr^{-1}. The enzyme is not associated with a particule since the activity remains in the supernatant of a high speed (20,000 × g) centrifugation. Immersion of the supernatant in boiling water for 10 min completely destroyed the activity, attesting to the enzymatic nature of the oxidation. The enzyme preparation could be stored for a week in the frozen state without loss of activity.

The amount of supernatant containing the enzyme used in each of the following experiments with ascorbic acid was such that it fell on the linear portion of the plot of enzyme concentration versus rate of oxygen uptake, with constant concentration of substrate.

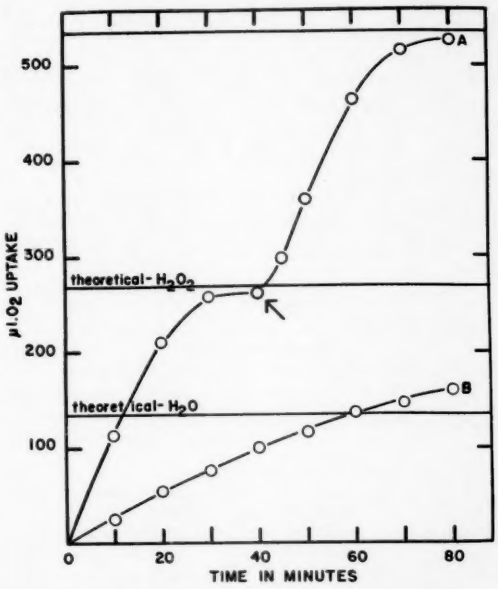

Fig. 1. The enzymatic oxidation of ascorbic acid in the presence of DDC. Curve A. Enzyme, 12 μ moles ascorbic acid + 1 μ mole DDC. Additional 12 μ moles ascorbic acid added at arrow. Curve B. Enzyme and 12 μ moles ascorbic acid.

[5] The pure compound was generously supplied by Dr. Eli Seifter of the Monsanto Chemical Company, Dayton, Ohio.

This eliminated the possibility of unsaturation of the enzyme with substrate; substrate saturation of this enzyme is necessary for valid inhibitor experiments and comparison of rates.

Effect of Inhibitors on the Oxidation of Ascorbic Acid: It is apparent that the oxidation of ascorbic acid by preparations of Physarum is not mediated through a typical ascorbic acid oxidase. The action of three powerful copper complexing agents, DDC, 8-hydroxyquinoline, and ethylenediamine tetra acetic acid, is shown in figure 2. Inhibition by these compounds, which would usually characterize a copper enzyme, does not occur in any case. Attention must be drawn to the striking stimulation produced by the addition of small amounts of DDC, which catalyzes the reaction to completion in about 30 min. This stimulation with DDC will be dealt with in later sections as one of the characteristic reactions of this enzyme.

The participation of other metal oxidases in this oxidation is also negated (table I). The observed stimulations with cyanide, azide, sulfide, and carbon monoxide are rare in the usual oxidase systems. Whereas the great stimulation of oxygen uptake with hydrogen sulfide may be ascribed to the reduction of the dehydroascorbic acid back to ascorbic acid, the stimulations due to the other compounds still remain obscure.

Mandels (20, 21) has recently described an enzyme from the spore surface of Myrothecium which oxidizes ascorbic acid and is resistant to cyanide, azide, sulfide, and DDC, with some stimulation with these compounds. This enzyme is inhibited by D-isoascorbic acid. In an experiment of the present work, it was demonstrated that this compound was oxidized by the Physarum enzyme to the same extent as ascorbic acid, thus exhibiting a less specific nature of this enzyme. These observations serve to differentiate these two enzymes.

Substances which inactivate -SH groups were utilized to characterize the enzyme further. No inhibition of the oxidation of ascorbic acid was observed

Table I

Effect of Respiratory Inhibitors on the Oxidation of Ascorbic Acid

Inhibitor	Conc	% Stimulation	
		Asc. acid *	Asc. acid + DDC †
KCN	0.001 M	38.6	65.3
KCN	0.0001 M	27.4	...
Na_2S	0.001 M	51.5	25.0
NaN_3	0.001 M	11.0	6.5
CO **	95 % CO; 5 % O_2	35.8	...

* Calculated from O_2 uptakes/hr, neglecting the first 10 min manometer readings.

** With controls having a gas phase of 95 % N_2; 5 % O_2 in the light and dark.

† Calculated from the overall O_2 uptake at the end of the reaction.

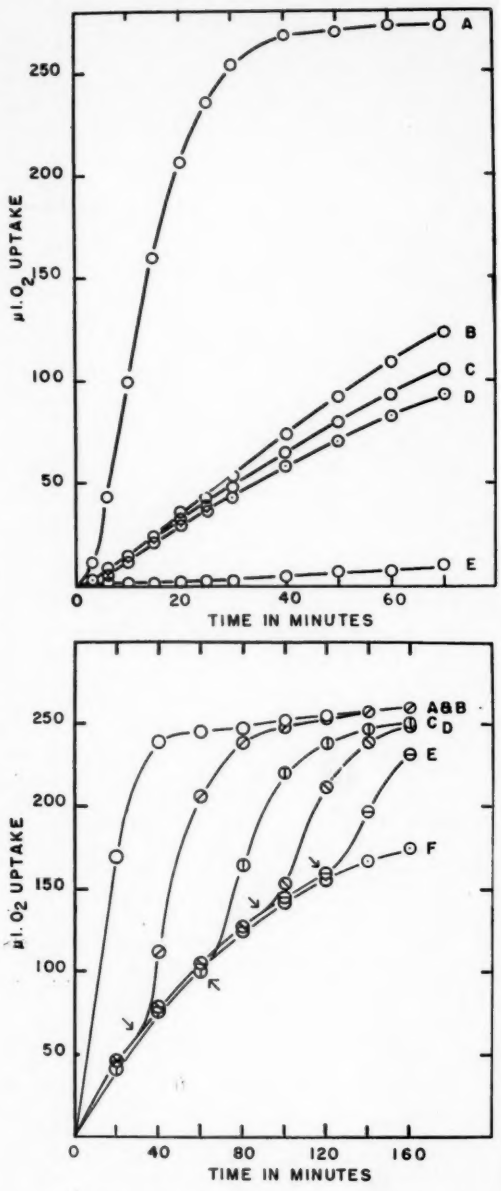

Fig. 2 (*above*). The effect of copper inhibitors on the enzymatic oxidation of ascorbic acid. Curve A. Enzyme, 0.006 M ascorbic acid + 0.0001 M DDC. Curve B. Enzyme, 0.006 M ascorbic acid + 0.001 M 8-hydroxyquinoline. Curve C. Enzyme, 0.006 M ascorbic acid + 0.001 M ethylenediamine tetra acetic acid (Sequestrene). Curve D. Enzyme and 0.006 M ascorbic acid. Curve E. Enzyme alone.

Fig. 3 (*below*). Delayed addition of DDC to the enzyme-ascorbic acid system. Enzyme, 0.006 M ascorbic acid, 0.0005 M DDC added at arrows. Curve A. DDC

TABLE II

THE EFFECT OF SULFUR COMPOUNDS ON THE ENZYMATIC OXIDATION OF ASCORBIC ACID

COMPOUND	M CONC	EFFECT WITH	
		ASC. ACID	ASC. ACID + DDC
Cysteine	0.001	None	Inhibits †
Glutathione, reduced	0.001	None	Inhibits †
Thioglycolic acid	0.001	None	None
2,3-Dimercapto propanol (BAL)	0.0001	None	None
2,3-Dimercapto propanol (BAL)	0.001	Lag *	Lag **
Cystine	0.001	None	None
Glutathione, oxidized	0.001	None	None
Tetraethylthiuram disulfide	(Solid)	Stimulates	.....
Thiourea	0.001	None	.....

* A lag of 40 min occurred, then the normal ascorbic acid oxidation curve was followed.

** A lag of 30 min occurred, then the catalyzed ascorbic acid level was obtained.

† Inhibits the catalyzing effect of DDC. The curves revert to the uncatalyzed type of curve.

with the alkylating agent, iodoacetate, while initial stimulation, but no inhibition, was obtained with the arylating agent, para-quinone. Para-chloromercuribenzoic acid, which forms mercaptides with thiol groups, slightly stimulates the oxygen uptake over the entire range. From those results the conclusion may be reached that the enzyme activity is not dependent upon any -SH groups which may be attached to the protein molecule. With respect to the stimulation of these latter inhibitors, two possible explanations are available. Either there is a -SH protein which inhibits the oxidation of ascorbic acid, or soluble -SH compounds are present which, when chemically transformed, no longer may function as oxidation inhibitors, but may function as oxidation promoters. To test the latter two possibilities, several thiol compounds were examined as to their action (table II). No inhibition of the oxidation of ascorbic acid could be observed with cysteine, reduced glutathione, thioglycollic acid, and BAL, and the possibility of -SH compounds inhibiting the oxidation of ascorbic acid seems to be without basis.

THE ROLE OF DDC: It has been observed (figs 1, 2) that a catalytic amount of DDC can stimulate the oxidation of ascorbic acid, since the addition of an equal amount of ascorbic acid at the end of the initial reaction results in an oxygen uptake as before. To further establish that the ascorbic acid is actually being oxidized, with or without the addition of catalytic amounts of DDC, advantage can be taken of the difference in ultra-violet absorption curves for ascorbic and dehydroascorbic acid. Ascorbic acid exhibits a peak of 245 mμ with metaphosphoric acid; however, dehydroascorbic acid has no absorption in this region. The procedure was as follows: a series of Warburg vessels were assembled with a reaction mixture of enzyme, ascorbate, and DDC; then placed in the bath, equilibrated, and the reactants added at zero time. At 0, 30, and 60 min reaction time, 0.5 ml of 25 % metaphosphoric acid was added. The contents of each vessel were transferred quantitatively to a centrifuge tube and centrifuged at 500 × g for 15 min. Ascorbic acid analyses were then determined on the supernatants of each on the Beckman DU spectrophotometer at 245 mμ. The results obtained show a definite decrease in optical density which is expected if ascorbic acid is being oxidized (table III). The effect of delayed addition of DDC on the oxidation of ascorbic acid is shown in figure 3. It can be observed that the later the addition, the greater the lag after each addition. However, after each addition, the reaction terminates at approximately the same oxygen level which, as will be shown later, approaches the theoretical oxygen level calculated for that amount of ascorbic acid.

The fact that the addition of ascorbic acid at the end of the reaction results in an oxygen uptake as before, indicates that the DDC is not destroyed during the course of the reaction and suggests its action may be that of a carrier. If the action of DDC is that of a carrier, then the addition of the disulfide, TTD, should give a similar stimulation. Such a stimulation is observed in figure 4. Therefore, the possibility exists that the DDC shuttles from the monosulfide to the disulfide form and back again during the reaction. It is observed that the acceleration due to TTD is not as prominent as that due to DDC. This may be attributed to the extreme insolubility of TTD, solid TTD being used in the reaction mixture. The problem of the insolubility of TTD led to the use of DDC exclusively in the following experiments.

The large amount of substrate oxidized by minute amounts of DDC in the presence of enzyme lends support to the idea that DDC has coenzyme-like properties. From figure 5, it may be seen that 0.2 μmoles of DDC can effectively catalyze the oxidation of ascorbic acid. The main effect of hundred-fold increase of DDC is that of increasing the slope of the initial oxygen uptake. If DDC takes some part in the direct oxidation in its reaction with ascorbic acid, 4 moles DDC would be needed to combine with one

TABLE III

THE OXIDATION OF ASCORBIC ACID AS FOLLOWED IN THE SPECTROPHOTOMETER

REACTION MIXTURE *	OPTICAL DENSITY AT		
	0′	30′	60′
Enzyme + ascorbic acid	.387	.252	.200
Enzyme + ascorbic acid + DDC	.425	.095	.055

* The blank consisted of boiled enzyme.

added at 0 min. Curve B. DDC added at 30 min. Curve C. DDC added at 60 min. Curve D. DDC added at 90 min. Curve E. DDC added at 120 min. Curve F. Control, ascorbic acid only.

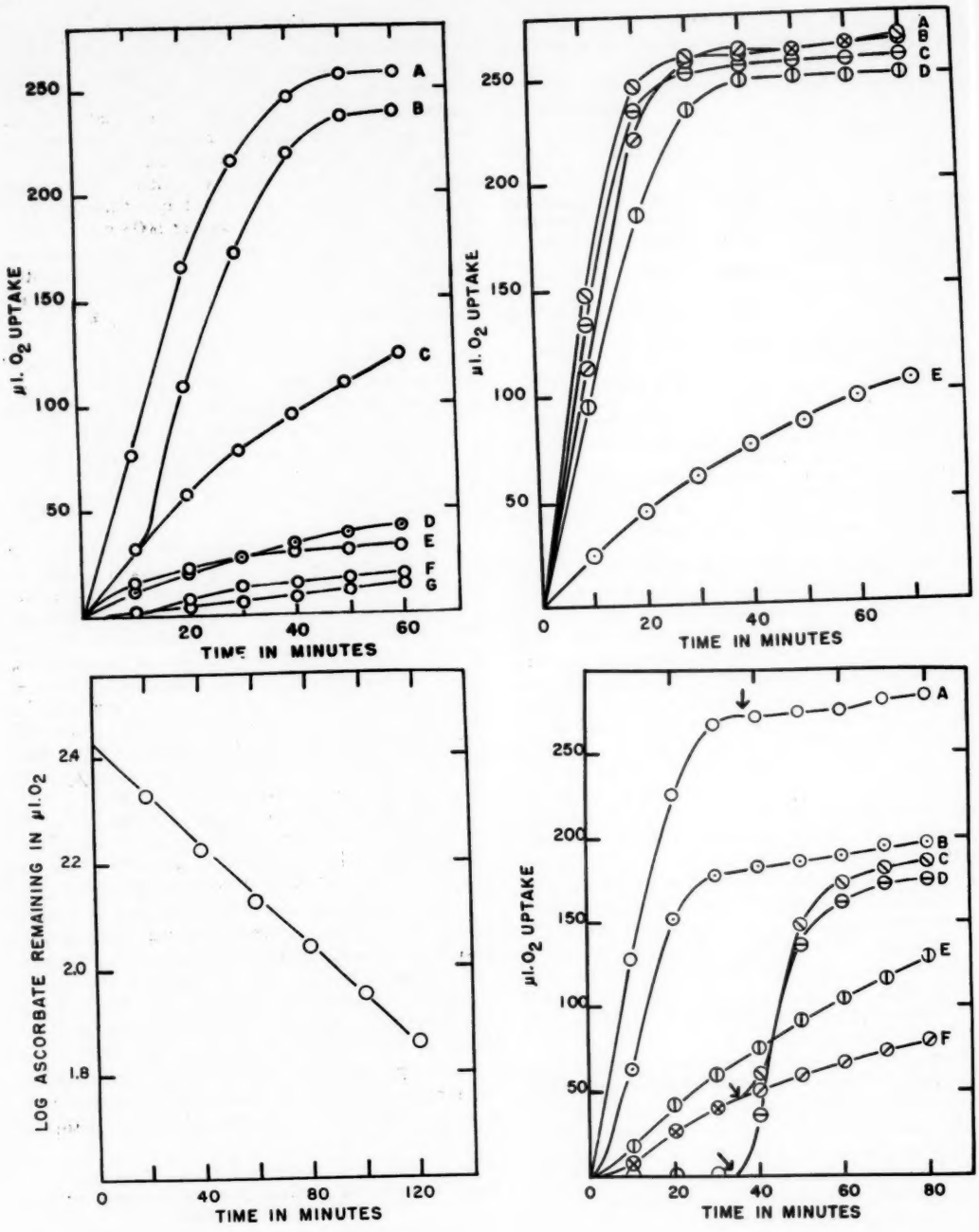

FIG. 4 (*upper, left*). The enzymatic oxidation of ascorbic acid in the presence of TTD and DDC. Curve A. Enzyme, 0.006 M ascorbic acid + 0.0001 M DDC. Curve B. Enzyme, 0.006 M ascorbic acid + solid TTD. Curve C. Enzyme and 0.006 M ascorbic acid. Curve D. Enzyme and 0.001 M DDC. Curve E. Enzyme and 0.0001 M DDC. Curve F. Enzyme and solid TTD. Curve G. Enzyme alone.

FIG. 5 (*upper, right*). The effect of catalytic amounts of DDC on the enzymatic oxidation of ascorbic acid. Curve A. Enzyme, 12 μ moles ascorbic acid and 24 μ moles DDC. Curve C. Enzyme, 12 μ moles ascorbic acid

mole O_2 to form H_2O, or 2 moles DDC to one mole O_2 for H_2O_2. Assuming the latter possibility:

24 μmoles DDC/12 μmoles O_2 = 269 μl O_2
0.2 μmoles DDC/0.1 μmole O_2 = 2.2 μl O_2

the reaction being:

$$2(CH_3CH_2)_2NCSSH + O_2 \longrightarrow (CH_3CH_2)_2NCSS\text{-}SSCN(CH_2CH_3)_2 + H_2O_2$$

The results show that this difference in the oxygen uptake does not exist, and that the action of DDC may well be that of a carrier. The effect of a ten-fold increase in the concentration of DDC in the absence of ascorbic acid results in no appreciable increase in oxygen uptake (fig 4).

The question of chelation must be considered as a possible mode of action for DDC, since this compound is a strong chelating agent for copper, cadmium, cobalt, lead, zinc, iron, and magnesium at physiological temperatures and pH (1). A possible explanation for its action here is that it is complexing some metal which in some manner prevents the oxidation of ascorbic acid. However, 8-hydroxyquinoline (2), and ethylene diamine tetra acetic acid (3) are also strong chelators under the same conditions. The negative results obtained with these latter compounds suggest that the action of DDC is not that of a chelator (fig 2).

Efforts to replace the stimulation effect of DDC with cysteine, reduced glutathione, thioglycollic acid and 2,3-dimercaptopropanol (BAL), and of TTD with cystine, or oxidized glutathione, failed (table II). Likewise the addition of coenzyme I, coenzyme II, and coenzyme A failed to replace DDC in the reaction mixture.

THE EFFECT OF INHIBITORS ON THE ASCORBIC ACID-DDC-TTD SYSTEM: Since the oxidation of ascorbic acid is stimulated by DDC, the effects of various inhibitors should be similar to those observed in the absence of DDC. Again, stimulations of varying degrees are obtained with the usual respiratory inhibitors (table I). In the DDC-ascorbic acid system plus these inhibitors the initial rates are approximately the same. However, some differences due to stimulations by these compounds are observed in the overall oxygen uptake after the stimulation due to DDC is completed. It may be that the only real stimulation of the overall oxygen uptake is that of cyanide. Azide stimulation is practically negligible, and the hydrogen sulfide stimulation can be ascribed to the reduction of dehydroascorbic acid. Stimulation of the oxidation of ascorbic acid with cyanide has been observed in wheat by Waygood (30). Although he observed a cyanide-sensitive oxidation of ascorbate, the addition of coenzyme I, or malate, to the reaction gave a stimulation apparently due to cyanide. He ascribes the action of cyanide as being a ketone fixative, or removing an inhibitor of the oxidation of ascorbate. Although he did not enlarge on the former explanation, it may be that the cyanide ties up the oxalacetate which is an inhibitor of malic dehydrogenase. It is possible that an analogous situation exists here with cyanide tying up an inhibitor.

Compounds, which combine or react with thiol groups, can be utilized in elucidating functional groups and sites on the DDC molecule. Para-chloromercuribenzoic acid and para-quinone did not affect the action of DDC at a concentration of 0.0001 M, but completely inactivated any catalysis by this substance at a concentration of 0.001 M. This is a complete inactivation of the effect of DDC, since the 0.001 M curves are most similar to those of the oxygen uptake due to ascorbic acid alone, where the DDC is absent. These results may be explained on the basis that the concentration of DDC is 0.0005 M, which is halfway between the two concentrations of inhibitor used. Thus, in one case, the -SH group may be completely combined with *p*-quinone and *p*-chloromercuribenzoic acid, but in the other it may be incompletely combined, leaving some -SH groups available for stimulation. This evidence lends credence to the possibility that the -SH group is an important functional group on the catalyzing molecule. The ineffectiveness of iodoacetic acid up to concentrations of 0.01 M does not support this view. However, this compound may react slowly with DDC, since the inhibitor was in contact with DDC only from zero time. It is also observed that cysteine and reduced glutathione were successful in completely blocking the stimulatory effect due to DDC, whereas BAL and thioglycolic acid were inactive (table II). In this connection it is observed that the disulfides, cystine and oxidized glutathione do not compete with DDC, which again amplifies the importance of the thiol group.

THE FORMATION OF H_2O_2: St. Huszak (14), in 1937, and subsequently others (27), cited experimental evidence that H_2O_2 was produced during the enzymatic oxidation of ascorbic acid by ascorbic acid oxidase. Steinman and Dawson (24) reinvestigated this alleged formation of H_2O_2, and proved conclusively that H_2O and not H_2O_2 was an end product of this oxidation. The aerobic oxidation of ascorbic acid as catalyzed by copper ion results in the formation of H_2O_2 as an end product (9, 31, 32). Indirect evidence, supplied from three independent approaches to

and 8 μ moles DDC. Curve B. Enzyme, 12 μ moles ascorbic acid and 4 μ moles DDC. Curve D. Enzyme, 12 μ moles ascorbic acid and 0.2 μ moles DDC. Curve E. Enzyme and 12 μ moles ascorbic acid.

FIG. 6 (*lower, left*). The enzymatic oxidation of ascorbic acid. Enzyme and 12 μ moles ascorbic acid.

FIG. 7 (*lower, right*). The addition of catalase to the enzyme-ascorbic acid-DDC system. Ascorbic acid at 0.006 M, DDC at 0.0005 M, 4.32 Keil units catalase added. Curve A. Enzyme, ascorbic acid and DDC, catalase added at arrow. Curve B. Enzyme, ascorbic acid and DDC, catalase added at 0 time. Curve C. Enzyme, ascorbic acid and catalase, DDC added at arrow. Curve D. Enzyme, catalase and DDC, ascorbic acid added at arrow. Curve E. Enzyme and ascorbic acid. Curve F. Enzyme, ascorbic acid and catalase added at 0 time.

the problem, point to the production of H_2O_2 as an end product of the enzymatic oxidation of ascorbic acid in Physarum. These approaches are briefly, (1) a manometric analysis, (2) a kinetic analysis, and (3) the use of catalase in the reaction mixture.

For the complete oxidation of ascorbic acid to dehydroascorbic acid, the required oxygen to ascorbate ratio for the formation of H_2O is one-half molecule O_2 per molecule of ascorbate. The ratio for the formation of H_2O_2 would be one molecule of O_2 to one molecule of ascorbate. The acceleration produced by the addition of DDC to the ascorbic acid-enzyme reaction mixture served to stimulate the reaction to completion, as was shown above. Attention must be drawn to the fact that the overall oxygen uptake at the completion of the DDC catalyzed reaction closely compares with that theoretical oxygen uptake required for the formation of H_2O_2 as an end product (fig 1). The oxidation due to ascorbic acid alone eventually approaches this value as time progresses. The oxygen uptake has never been observed to quite reach the theoretical, but it is always about 5 to 15 μl O_2 less. This is probably due to the weak catalase activity which is present in the enzyme preparation.

A striking feature of the typical copper containing ascorbic acid oxidase is that the enzyme exhibits a marked inactivation during the reaction which it catalyzes (23). It is observed (fig 1) that little if any inactivation of the enzyme occurred at least with this amount of enzyme, since the second addition of ascorbic acid is oxidized at approximately the same rate and extent as the first. It is apparent that the oxygen uptake obtained at the cessation of the reaction is not due to any enzyme inactivation phenomenon.

In a reaction of the first order, the reaction proceeds with the decrease of only one substance. If such a relation exists when ascorbic acid is being oxidized, a plot of $\log(C_o - C)$ versus time should yield a straight line. $\text{Log}(C_o - C)$, or ascorbic acid remaining at time t, was converted to values in terms of μl O_2. A semilogarithmic plot of ascorbic acid remaining in terms of μl O_2 versus time produced a straight line, if and only if, the oxidation of ascorbic acid was considered as forming H_2O_2 as an end product (fig 6). That is, the remaining ascorbic acid at time t was converted to μl O_2 at the theoretical ratio of one molecule ascorbic acid per one molecule of O_2. It must be emphasized here that this kinetic analysis applies only to the ascorbate systems where no DDC or TTD is added. With added DDC or TTD, the system becomes dependent upon another component, and by definition the reaction becomes one of higher order and complicated in its analysis. Several attempts with second order equations, using the same analytical technique above, failed to show any correlation. Reactions which are truly unimolecular in mechanism are rare; however, the first order equation also applies to many pseudo-unimolecular reactions (11). Actually, the pseudo-unimolecular condition exists in the above reaction since it depends upon the concentration of oxygen, which is present in such an

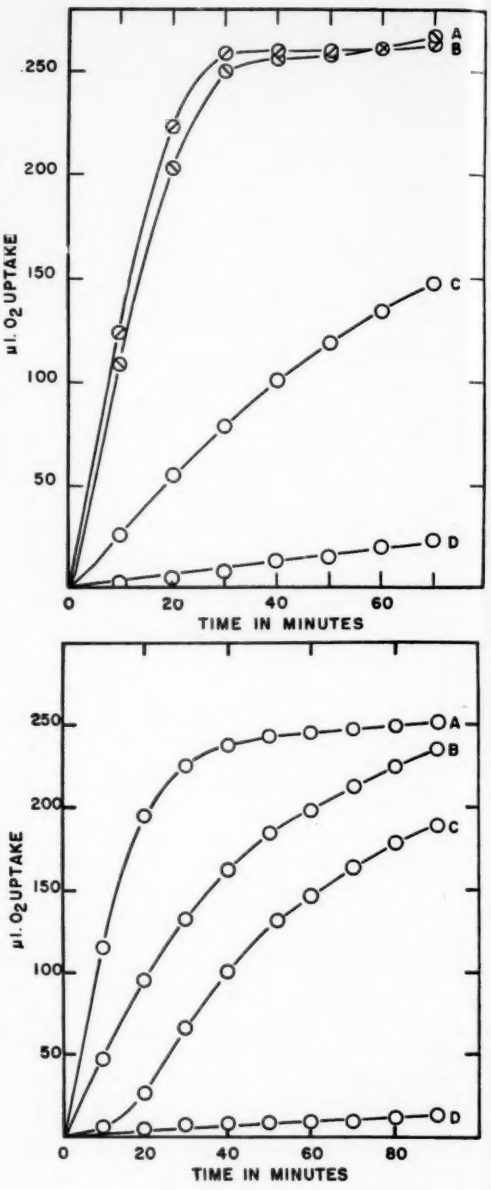

FIG. 8 (*above*). The effect of dialysis on the enzymatic oxidation of ascorbic acid. Ascorbic acid 0.006 M, DDC 0.0005 M. Curve A. Undialyzed enzyme + ascorbic acid and DDC. Curve B. Dialyzed enzyme + ascorbic acid and DDC. Curve C. Undialyzed enzyme + ascorbic acid. Curve D. Dialyzed enzyme + ascorbic acid.

FIG. 9 (*below*). Effect of added concentrate on the enzymatic oxidation of ascorbic acid. Ascorbic acid 0.006 M, DDC 0.0002 M, dialyzed enzyme preparation. Curve A. Enzyme, ascorbic acid and DDC + conc.

excess that the change in concentration during the reaction is small and may be neglected here. An analysis of this sort, although not valid in a kinetic sense, is utilized here only to demonstrate the possible formation of H_2O_2.

The use of added catalase in the ascorbate oxidation would lend evidence, pro or con, concerning the formation of H_2O_2. It is seen in curve B (fig 7) that the addition of catalase to the reaction mixture at zero time produced a decrease in the oxygen uptake. This suggests that H_2O_2 was formed as an end product since the catalase would evolve oxygen and would have the effect of decreasing the overall oxygen uptake. That a decrease of one half of the oxygen uptake was not obtained indicates that H_2O_2 may participate in other reactions involving internal components in the extract but which do not involve a gas exchange. This idea is supported by curve A where catalase was added at the end of the reaction. The absence of a gush of oxygen at this point in the reaction points to the absence of H_2O_2, which may have reacted in some other manner. Curves B, C, and D reveal that the delayed addition of either DDC or ascorbate to the system does not seriously affect the action of catalase. Curves E and F further attest to the formation of H_2O_2 in the oxidation of ascorbate in the absence of DDC, since catalase action decreased the overall oxygen uptake.

The direct determination of H_2O_2 enzymatically (19) using tolidine and potato juice, a source of peroxidase, and by chemical titration with thiosulfate (24) yielded negative results. This is to be expected since apparently the H_2O_2 once formed is very unstable in the reaction mixture and reacts instantaneously in an unknown manner.

One cannot ignore the indirect evidence reported here for the production of H_2O_2 as an end product in this oxidation. The failure to demonstrate directly the formation of H_2O_2 is presumed to be a consequence of an impure enzyme preparation and cannot be considered in this case as conclusive evidence.

The Effect of Dialysis: The effect of dialysis on the enzyme preparations of Physarum markedly decreased the ascorbic acid oxidizing activity (fig 8). One half of an enzyme preparation was dialyzed at 4° C against 0.02 M KH_2PO_4-Na_2HPO_4 buffer, pH 7.2, for 24 hours, and the other half was kept at the same temperature for the same length of time. It can be observed that although the ascorbic acid activity was decreased upon dialysis, the addition of DDC catalyzed the complete oxidation of ascorbic acid as before. It appears as if DDC serves as a model for some dialyzable compound which is necessary for the oxidation of ascorbic acid.

If such a dialyzable factor actually participates in this oxidation, it is only necessary to add back this dialysate and activity will be restored. Such an experiment was performed as follows: 80 gm of plasmodia were homogenized and dialyzed against one liter of distilled water at 4° C for 36 hours. This yellow dialysate was concentrated under vacuum to approximately 10 ml (the temperature did not rise above room temperature), and aliquots were added back to the dialyzed reaction mixture. Upon concentration, the solution, which was originally yellow, turned deep black. The concentrate restored ascorbic acid activity to the dialyzed enzyme to a greater extent than that due to DDC (fig 9). When both DDC and concentrate were present together, the stimulation was greater, and a more typical catalyzed curve was produced. The difference in stimulations produced by DDC and the concentrate in this experiment appears to be one of degree rather than kind, since it is observed that when a stronger concentration of DDC is added (fig 8) the curve produced is similar to that of curve A in figure 9. Whether the concentrate contains a compound identical with DDC or similar to it in structure is a matter for further experimentation, since a preliminary attempt to identify this compound with paper chromatographic methods (25) failed to give any information.

The yellow color of the dialysate from extracts of Physarum is due to the presence of a pigment or pigments which upon spectrophotometric analysis yielded an absorption spectrum with two peaks; one at 260 mμ, and a relatively broad peak at 370 mμ. This pigment is at present unidentified, but appears to be related to the flavins, since two of the three flavin peaks are present.

Other Oxidizing Systems: Attempts to replace ascorbic acid in the DDC-ascorbic acid system failed, since the addition of tyrosine, catechol and hydroquinone produced no additioned O_2 uptake over that of the endogenous, with or without the presence of DDC. These results indicate that these compounds are not utilized by the ascorbic acid enzyme in agreement with the evidence found with the Myrothecium enzyme (20) and purified preparations of ascorbic acid oxidase (16, 28). Further, it is apparent that this fraction contains no tyrosinase, catecholase, or laccase.

Ascorbic acid and hydroquinone have been utilized in many cytochrome-cytochrome oxidase systems to keep the cytochrome in the reduced state, thus being the indirect cause of the oxygen uptake. The fact that no oxygen uptake results from hydroquinone in these experiments and the insensitivity of the oxygen uptake due to ascorbic acid to the respiratory inhibitors imply the lack of a cytochrome oxidase system in this fraction, although it is present in the particulate fraction (29). A previous report of the measurement of the cytochrome oxidase activity in this organism (13) using ascorbic acid as the reducing agent for the cytochrome must be questioned since the above evidence shows that the oxidation of ascorbic acid is mediated through another pathway.

Curve B. Enzyme, ascorbic acid + conc. Curve C. Enzyme, ascorbic acid + DDC Curve D. Enzyme and ascorbic acid.

Discussion

Resistance, and even stimulation, of the enzyme to the usual metal oxidase inhibitors plus the unique catalysis of ascorbic acid oxidation by DDC and TTD and the possible formation of H_2O_2 serve to differentiate this enzyme from the typical copper ascorbic acid oxidase and the non-metallic ascorbic acid oxidase of Myrothecium.

Several experimental facts lend credence to the idea that DDC and TTD function as model substances for a natural carrier or cofactor. The stimulation of the oxidation of ascorbic acid with catalytic amounts of both DDC and TTD indicates that a shuttling of the -SH to the -S-S- form and back again occurs. When a second amount of ascorbic acid is added to a completed ascorbic acid-DDC reaction, there results an oxygen uptake as before. Again this shows that the DDC is not destroyed but may still participate in its shuttling action. That a natural cofactor or carrier is present is evident from the dialysis experiments in which the concentrated dialysate can restore ascorbic acid oxidizing activity to the enzyme. It follows that the "turn-over" number of DDC in the enzymatic oxidation of ascorbic acid is low (1.5 molecules per min per molecule of O_2) as compared to that of known carriers, e.g., Co I has a turn-over number of 10^4 per molecule enzyme (5) and cytochrome c can be oxidized and reduced 4,000 times a minute (10). This is not surprising, since DDC again may not be the natural cofactor of the system and as such is not efficient in its action as a carrier. Since the identity of the natural cofactor and the molecular weight of the enzyme are unknown, the calculation of a turn-over number for the natural carrier must await further experimentation.

Summary

An atypical enzymatic oxidation of ascorbic acid is present in the supernatant from a high speed ($20{,}000 \times g$) centrifugation of the homogenates of *Physarum polycephalum*. The enzyme is resistant to cyanide, azide, sulfide and carbon monoxide as well as metal chelators and -SH enzyme inhibitors. The oxidation of ascorbic acid is stimulated by catalytic amounts of diethyldithiocarbamate (DDC) and tetraethylthiuram disulfide (TTD). Indirect evidence indicates the formation of hydrogen peroxide as an end product of this oxidation. Dialysis markedly inactivates the ascorbic acid oxidizing ability of the enzyme. The addition of DDC or concentrated dialysate results in the restoration of activity. The possibility of DDC and TTD acting as model substances for a natural unknown carrier is discussed.

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OXIDATION OF 2,3′,6-TRICHLOROINDOPHENOL BY THE LIPOXIDASE SYSTEM[1,2]

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In 1949 Smith and Stotz (11) found that homogenates from several plant sources were able to catalyze directly the oxidation of the leuco (reduced) form of the dye 2,3′,6-trichloroindophenol (2,6-dichlorobenzeneoneindo-3′-chlorophenol) to the blue form, a reaction involving the removal of hydrogen, and the enzyme system was called "dye oxidase." The nature of the enzyme was not completely elucidated, but it was concluded (11) that "dye oxidase" was distinct from peroxidase, catechol oxidase and cytochrome oxidase, although each of these enzymes in the presence of its substrate was shown to be able to oxidize the leuco dye (10, 11, 12). Balls, Axelrod and Kies (1) had previously shown that a closely related indophenol dye could be oxidized through the lipoxidase system; in fact it has been known for some time that a variety of dyes and other substances may be oxidized by a coupled reaction if they are present when lipoxidase is catalyzing the oxidation of its substrates—linoleic, linolenic and arachidonic acids and their esters—by atmospheric oxygen (see (3) for a review). This suggested the possibility to us that such a system might contribute to "dye oxidase" activity and an investigation was undertaken with the results described below.

MATERIALS AND METHODS

The procedure of Smith and Stotz (11) was followed to obtain the dye solutions; the dye was reduced with gaseous hydrogen in the presence of palladium, and was used immediately after reduction. Linoleic acid was emulsified with gum ghatti (2). (The gum ghatti solution, in the quantities used, did not affect the rate of autoxidation of the reduced dye, nor did the blue dye fade in its presence.) Soybean lipoxidase prepared according to Cosby and Sumner (1a), was purchased from the Worthington Biochemical Corporation, Freehold, New Jersey; the solid was dissolved in water (5 to 10 mg/ml) as enzyme solution. Catalase was obtained from Bios Laboratories, New York. Reactions were performed at room temperature in cuvettes of one cm light path, and a Beckman Model B spectrophotometer was used to follow changes in the optical density of the dye at 645 mμ. (For ultra-violet measurements, silica cuvettes were used with a Beckman Model DU spectrophotometer.)

RESULTS

The leuco dye was found to be oxidized by soybean lipoxidase plus linoleic acid, but neither was effective alone. Figure 1 shows that when lipoxidase was added to the reduced dye containing 0.3 ml of linoleic acid emulsion, a rapid production of the blue form of the dye occurred (curve 6); the rate of the reaction was proportionately lower with smaller amounts of linoleic acid (curves 4 and 5). However, after the first two to three minutes of the reaction, the dye began to fade (curves 4, 5, and 6), and this loss of color was more rapid with increasing amounts of linoleic acid. This fading (which was distinct from the reduction of the blue dye, since the dye was permanently bleached and the color could not be regenerated with the use of mild oxidizing agents), suggested that a secondary reaction was superimposed upon the initial oxidation of the leuco dye. By employing the blue (oxidized) form of the dye as the starting material, it was found that neither linoleic

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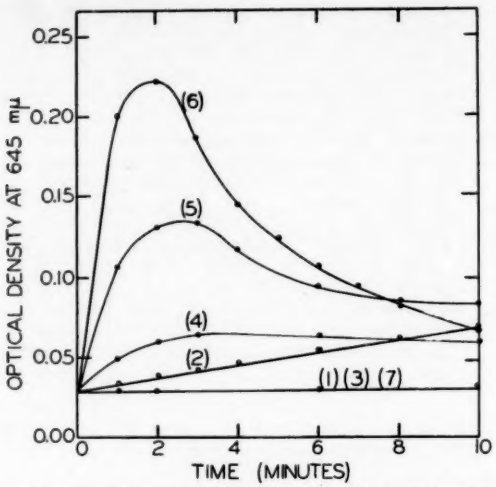

FIG. 1. The effect of soybean lipoxidase (5 mg/ml) and linoleic acid emulsion (0.025 ml/ml) upon the leuco (reduced) dye. 3.0 ml of leuco dye solution (pH 6.5, prepared according to Smith and Stotz (11)) and 0.2 ml of lipoxidase solution were used per cuvette; the enzyme solution was added last at zero time. Curves: 1. Dye. 2. Dye + lipoxidase. 3. Dye + 0.3 ml linoleic acid. 4. Dye + lipoxidase + 0.1 ml linoleic acid. 5. Dye + lipoxidase + 0.2 ml linoleic acid. 6. Dye + lipoxidase + 0.3 ml linoleic acid. 7. Dye + boiled lipoxidase + 0.3 ml linoleic acid.

acid nor soybean lipoxidase alone had any effect upon the color, but that the addition of both of these together to the blue dye caused a rapid and permanent fading. By the use of Thunberg technique, it was found that oxygen was required for both the enzymatic oxidation of the leuco dye and for the enzymatic fading of the blue form.

Although the fading was a complicating feature of the reaction, it was clear that lipoxidase and its substrate could act as "dye oxidase." Therefore it was of interest to find if the lipoxidase system existed in the tissue extracts used by Smith and Stotz. For this purpose potato was selected, since this exhibited especially high "dye oxidase" activity (11). Potato homogenates which were shown to possess thermolabile "dye oxidase" activity, catalyzed the oxygen absorption of linoleic acid, and the optical density at 234 $m\mu$ of methanol extracts of the reaction mixture (prepared according to the procedure of Holman (2)) increased during the reaction and paralleled the oxygen consumption; this increase in optical density has been shown to be associated with the enzymatic oxidation of lipoxidase substrates (2, 13). The presence of substrates of lipoxidase in the potato was suggested in an experiment in which the acidified homogenate was extracted with ether; the residue remaining after evaporation of the ether was emulsified with phosphate buffer and was found to absorb oxygen in the presence of soybean lipoxidase. This demonstration of the lipoxidase system in potato confirms the results of Kirsanova (4), who found "carotene oxidase" in homogenates from this tissue. ("Carotene oxidase" was later renamed lipoxidase, since the oxidation of carotene was shown to be due to a coupled reaction brought about by lipoxidase and its substrates—for a review see Holman and Bergström (3).)

The possibility that enzyme systems other than lipoxidase can act as "dye oxidase" in any particular extract is not excluded by the above experiments. Smith, Robinson and Stotz (10) have shown that peroxidase in the presence of its substrate oxidized the leuco dye and demonstrated the existence of this enzyme in potato homogenates. In considering the identity of "dye oxidase," Smith and Stotz (11) suggested that peroxidase was not involved; however, Dr. F. G. Smith (personal communication) indicated that "dye oxidase" activity could be inhibited by cyanide and azide, and might be attributed to peroxidase, if a source of hydrogen peroxide were present. In the present investigation, it was found that when leuco dye oxidation occurred in the presence of soybean lipoxidase and linoleic acid, as shown in figure 1, addition of catalase or 0.01 M cyanide or azide had virtually no effect. This eliminated the possibility that a very feeble peroxidase activity, shown by colorimetric methods to be present in the soybean lipoxidase preparation, was involved in the oxidation. On the other hand, when leuco dye oxidation was catalyzed by potato homogenate ("dye oxidase" action), application of 0.01 M cyanide or azide inhibited the development of the blue color by 60 to 80 %, and catalase reduced the activity by 40 %. Thus although the lipoxidase system is present in potato, it appears that peroxidase plays a major role in "dye oxidase" activity of homogenates from this tissue. In addition, the operation of a distinct "dye oxidase" is not ruled out. The elucidation of the relative importance of the different oxidizing systems in extracts from other tissues would require further work.

The fading of the blue (oxidized) dye, observed with the soybean lipoxidase system was also observed when potato homogenates were used as the source of lipoxidase. The addition of both potato homogenate and linoleic acid together to the blue dye caused a rapid and permanent fading, which was unaffected by the addition of cyanide, azide or catalase.

DISCUSSION

Although both the lipoxidase and peroxidase systems may act as "dye oxidase," only lipoxidase in the presence of added substrate was found to cause the fading of the blue (oxidized) dye. It is therefore apparent that if lipoxidase and its substrate are present in any given tissue extract, they would interfere in an enzyme assay in which the dye was used. This factor would seem to weigh against the use of the dye in colorimetric enzyme assays.

The dye has no metabolic significance per se, but it is interesting to note that the reduction of the di-

chloro derivative (the trichloro derivative was used here) by succinate and succinate dehydrogenase has been developed as an assay method for the dehydrogenase (7). The present investigation of the oxidation of the reduced dye through the lipoxidase system indicates that the dye might serve in a model enzyme system as a carrier in the oxidation of succinate by atmospheric oxygen through the lipoxidase system. The fact that both cysteine and reduced glutathione may be oxidized to the corresponding disulfides through the lipoxidase system from pea seeds (5) focuses attention upon the possible importance of lipoxidase as a terminal oxidase in plant respiration. Thus, with glutathione reductase (6), reduced triphosphopyridine nucleotide might be oxidized by oxygen through the lipoxidase system; a similar sequence of reactions may be visualized for the oxidation of reduced diphosphopyridine nucleotide by atmospheric oxygen through cystine reductase (8), and the lipoxidase system.

Summary

The lipoxidase enzyme system from plant sources is able to catalyze the oxidation of the leuco (reduced) form of the dye 2,3′,6-trichloroindophenol to the blue form and may thus contribute to the activity called " dye oxidase " by Smith and Stotz. It appears that in potato extracts peroxidase systems also contribute to the " dye oxidase." A property peculiar to the lipoxidase system is that it causes the permanent oxidative fading of the blue form of the dye.

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INFLUENCE OF pH ON 2,4-DICHLOROPHENOXYACETIC AND ACETIC ACID ACTIVITY IN CHLORELLA [1,2]

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The principal effect of external pH on the activity of auxins and other weak organic acids is to regulate the degree of their dissociation (6, 20, 22). The importance of this effect lies in the fact that only the undissociated molecules of such acids appear to readily penetrate the plasma membranes of plant cells and to reach an equilibrium condition in which the concentration within the cell approximates that of the external bathing medium (6). It would therefore appear that the effects of any compound of this type on the functioning of a living cell should be directly related to the concentration of undissociated molecules in the bathing medium. However, Smith et al (21) demonstrated that the concentration of undissociated molecules producing a standard effect (LD 50 for spore germination of *Colletotrichum circinans*) was not constant over a wide pH range.

The question of the relative importance of the undissociated acid molecules and the ions of these compounds in the physiological responses of plants has recently been the subject of investigations by Simon and Blackman (20), Simon and Beevers (18, 19), and Blackman and Robertson-Cuninghame (3). They performed experiments and recalculated data of other workers to determine the concentration of undissociated molecules in the external medium that would give a standard response at several pH levels.

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Results indicated that below the pK of the acid being tested the concentration of undissociated acid required for a standard response did not vary greatly, but at progressively higher pH levels it appeared that the concentration of undissociated molecules required in the bathing medium was progressively less. These data were interpreted as indicating that the whole response could not be attributed to the undissociated molecules, alone, but that part of the response must be due to the ions.

In a previous publication on the inhibition of photosynthesis in *Chlorella pyrenoidosa* by 2,4-dichlorophenoxyacetic acid (2,4-D) (25) data were presented in which almost the complete range of inhibition of photosynthesis was achieved with a single concentration of 2,4-D by varying the pH of the solution or by selecting a single pH and varying the concentration of 2,4-D. However, insufficient data were available to plot the concentration of undissociated molecules required for a standard response over a wide pH range. Such data were subsequently obtained and are presented here. Because of the limited solubility of 2,4-D it was not possible to extend the study over the desired pH range. Additional studies were made using acetic acid. This acid was suitable because of its high solubility in water and its inhibitory effects on photosynthesis.

Materials and Methods

Chlorella pyrenoidosa Chick (Emerson's strain, kindly provided by Dr. David Appleman) was used as the test plant. Cells were produced in a glass tube 1.25 meters long and 37 mm in diameter, using a nutrient solution containing 0.025 M KNO_3, 0.02 M $MgSO_4$, 0.018 M $KHPO_4$, 0.001 M $CaCl_2$, and the following microelements: B, Mn, Zn, Cu, Mo, and Fe as tartrate. Air scrubbed through an activated carbon filter and water was bubbled through the solution to provide CO_2 and agitate the solution, keeping the cells in suspension. Illumination was provided by two 30-watt fluorescent lamps, one mounted on each side of the culture tube at a distance of about 25 mm. Aluminum reflectors were used to increase the light intensity. Cold air was passed between the fluorescent tubes and the culture tube to keep the solution between 25° and 30° C. The solution was inoculated with cells grown in pure culture on agar. After 3 days of growth in the nutrient solution under these conditions the density of cells was sufficient for determinations of photosynthesis and respiration in an illuminated Warburg respirometer which provided a light intensity of about 1200 fc at the level of the flasks (25).

Cell suspensions were centrifuged to separate the cells from the nutrient solution. A 10-ml aliquot of cell suspension containing approximately 20 mg (dry weight) of Chlorella cells was centrifuged for photosynthesis and 30 or 50 ml aliquots providing approximately five times as many cells were used for respiration measurements. The cells were resuspended in 3 ml of potassium citrate buffer (0.04 M) containing the necessary amounts of 2,4-D or acetic acid partially neutralized to the desired pH levels with KOH. These suspensions were transferred to the Warburg flasks.

Citric acid was previously tried for inhibition of photosynthesis and found to be without effect at the concentration used in the buffer. Eny (9, 10) showed that citric was one of the least effective of the organic acids tried for promoting growth or altering respiration of Chlorella.

Diethanolamine-carbonate buffer (14) was used in the two side arms and center well of the Warburg flasks to maintain a constant supply of CO_2 at about 1 % for photosynthesis. This buffer was composed of 60 ml diethanolamine, 45 ml 6 *N* HCl, 60 ml water, and 30 gm $KHCO_3$.

The bath was maintained at 25° C. After 30 minutes of equilibration the oxygen evolution during the ensuing hour was determined. This was followed by 30 minutes equilibration in the dark before measuring oxygen uptake. The oxygen evolution during the light period and the oxygen uptake during an equal dark period were combined to give a measure of true photosynthesis. Where respiration studies were made separately, the oxygen uptake during a 2-hour dark period was determined. Larger amounts of Chlorella were used to adjust the total oxygen uptake in the controls to an amount between 75 and 800 μl per

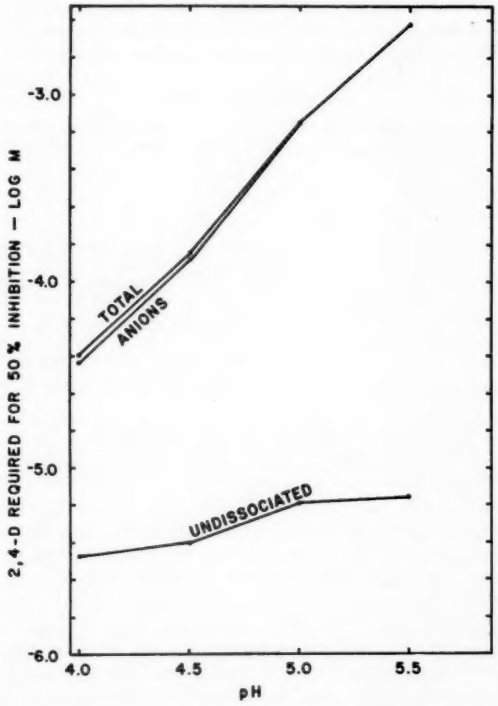

Fig. 1. Amount of 2,4-D required in bathing medium at various pH levels for 50 % inhibition of photosynthesis in *Chlorella pyrenoidosa*.

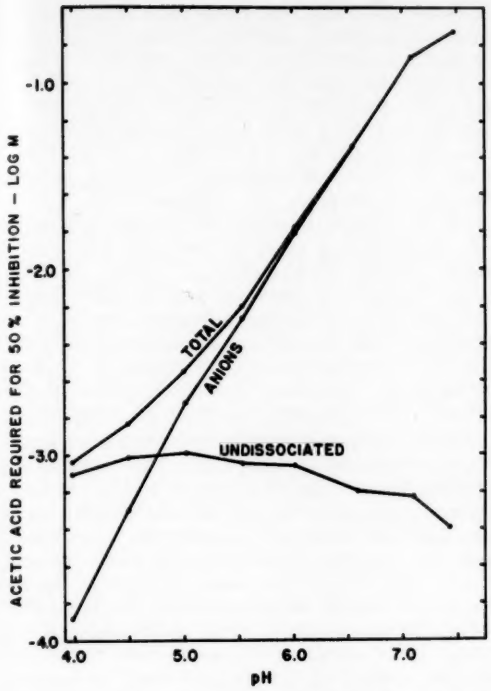

FIG. 2. Amount of acetic acid required in bathing medium at various pH levels for 50 % inhibition of photosynthesis in *Chlorella pyrenoidosa*.

hour. In such runs 10 % KOH was used in the side arms and center well to remove CO_2. In all cases the surface area of the solution in the center well was increased by insertion of a piece of pleated filter paper.

Calculations of undissociated acid were made by employing pH values determined after the runs, and pK 2.96 for 2,4-D (25), and pK 4.76 for acetic acid (8). The 2,4-D (provided by the Dow Chemical Co.) used was first recrystallized 3 times from hot water.

RESULTS

As in previous trials (25), the percentage inhibition of photosynthesis by 2,4-D over the middle range of inhibition fits straight lines when plotted against molar concentrations of undissociated 2,4-D better than it fits log molar concentrations. By using the regression equation calculated from the concentration series at each pH, the amount of undissociated 2,4-D required for 50 % inhibition of photosynthesis was determined for each pH studied. The latter ranged from pH 4.0 to pH 5.5 with points at half pH intervals. The total, the dissociated, and the undissociated 2,4-D required for 50 % inhibition at each pH are shown in figure 1. It is interesting to note that the amount of undissociated 2,4-D required for 50 % inhibition of photosynthesis in the Chlorella increased slightly over the range from pH 4.0 to pH 5.5.

The amount of undissociated acetic acid required for 50 % inhibition of photosynthesis in Chlorella was determined over the range from pH 3.97 to pH 7.42 (fig 2). Up to pH 5 there was a slight increase in the amount of undissociated acid required as the pH increased. Above pH 5.0 the amount of undissociated acid required for 50 % inhibition decreased slightly with increasing pH.

Respiration determinations on the Chlorella treated with 2,4-D were made only in conjunction with photosynthesis. Although the oxygen uptake under these conditions was relatively small, it showed that a stimulation of respiration was produced in the lower concentrations of 2,4-D at each pH.

A separate and more extensive measurement of respiratory stimulation was made using acetic acid. At each pH, concentrations of acid were chosen to permit calculation of the amount of acid required to produce 20 % stimulation of respiration.

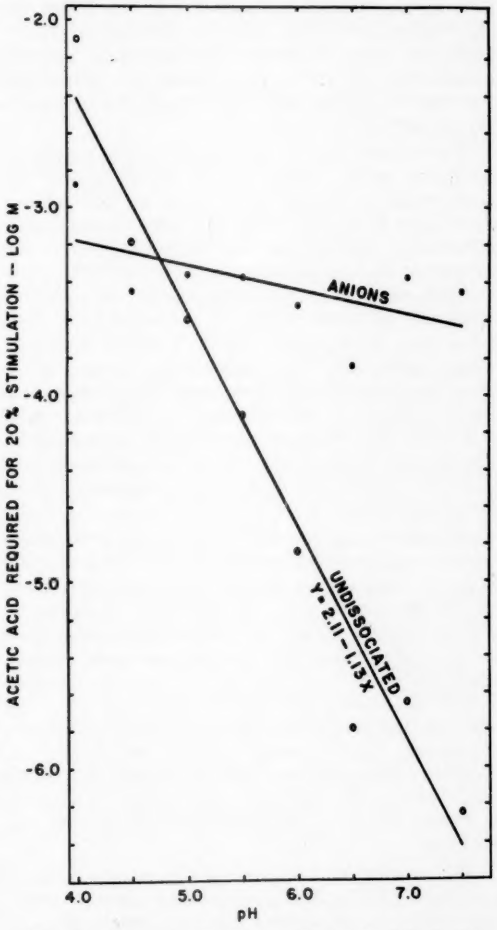

FIG. 3. Amount of acetic acid required in bathing medium at various pH levels for 20 % stimulation of respiration in *Chlorella pyrenoidosa*. For regression equation of anions, $Y = -2.66 - 0.128\,X$.

Instead of a nearly constant amount of undissociated acetic acid producing the 20 % stimulation at each pH, it was found that with each unit increase in pH the amount of undissociated acetic required was approximately one-tenth as much (fig 3). In other words, the standard 20 % stimulation of respiration was produced by a nearly constant anion concentration of acetate, regardless of pH.

Discussion

More than 200 times as much undissociated acetic acid as undissociated 2,4-D in the bathing medium was required to inhibit by 50 % the photosynthesis of *Chlorella pyrenoidosa*. Simon and Blackman (20) have suggested that a comparison of toxicities of different weak organic acids be made at pH values below the pK of the compounds concerned, so that there would not be a variation of toxicity with change in pH. In the present comparison pH 4 is below the pK of acetic acid (pK 4.76) but well above that of 2,4-D (pK 2.96). However, extrapolation of the lines in figure 1 to the pK of 2,4-D would not appreciably alter the relationship based on the undissociated molecules at pH 4.

The present data do not permit an explanation of the nature of the inhibition of photosynthesis, but they contribute to the information on the subject. Myers (13) found that at pH 4.5, 0.004 M acetic acid, or 0.0012 M butyric acid, or 0.0005 M caproic acid completely blocked photosynthesis in *Chlorella pyrenoidosa*. Since pH 4.5 is below the pK of each acid (n-butyric, pK 4.83; n-caproic, pK 4.85) (15), these values may be considered to be near the maximum toxicity (20). In the present study it was determined that the total acid estimated to produce complete inhibition of photosynthesis at pH 4.49 would be 3.02×10^{-3} M which agrees with the value given by Myers, particularly since he did not determine the least amount of acid that would produce complete inhibition.

Assimilation of acetate in plants has been abundantly demonstrated (1, 7, 9, 10, 11, 12, 13, 23, 24). None of these studies on the respiration of acetate related the activity to concentration of substrate. Myers (13) mentioned that concentration of acetate had little effect on oxygen uptake, but since no concentration limits were specified, it must be assumed that he was making determinations near or above the concentration producing maximum stimulation of oxygen uptake. In the present study it was found that, with increasing concentrations of acetate, the oxygen uptake increased rapidly to a maximum and then fell off rather slowly as the concentrations exceeded the optimum.

The problem of the effect of pH on the biological activity of weak acids has received considerable attention without a satisfactory solution. The earlier explanation, that the concentration of undissociated molecules in the external medium governed the activity, has become less acceptable in view of critical data and interpretations presented by Simon and Blackman (20) and by Simon and Beevers (19). Brian and Rideal (5) directed attention to the possible changes in proteins and protein complexes as being more pertinent than the concentration of undissociated acid. Although the proteins are undoubtedly of great importance, it must not be overlooked that the same range in pH has widely different effects on the change in toxicity of substances, depending, in most instances, on the pK of the substances (19).

In the present investigation extreme differences were noted between states of ionization and physiological responses over a wide pH range. Although in the inhibition of photosynthesis by acetic acid a reasonably close relationship existed between 50 % inhibition and the concentration of undissociated molecules in the external solution, the stimulation of respiration, on the other hand, was closely related to the external acetate ion over the whole pH range tested.

Similar extremes are to be found in the presentation of Simon and Beevers (19). One extreme, exemplified by the effect of hydrazoic acid on the inhibition of respiration of yeast (19, fig 3), shows less than a 10-fold change in concentration of undissociated acid required to produce a standard response over a 1,000-fold hydrogen-ion concentration change, whereas the amount of anion required between a 100- and 1,000-fold change for the same hydrogen-ion range. This relationship approaches that of a nearly constant amount of undissociated acid for a given response. The other extreme, exemplified by the effect of β-indoleacetic acid in the pea test and adapted from Bonner's work (4) by Simon and Beevers (19, fig 6), shows between 100- and 1,000-fold change in concentration of undissociated acid required to produce a standard response over a 1,000-fold hydrogen-ion concentration change, whereas the anion change is less than 10-fold. This relationship approaches that of a nearly constant amount of anion for a given response.

The inhibition of yeast respiration by hydrogen fluoride (19, fig 2) appears to be a combination of the relationships described above, in that from pH 3.2, the pK of hydrogen fluoride, to pH 5 the relationship is one of a constant amount of undissociated acid for the response, and above pH 5 the relationship is one of a nearly constant amount of anion.

These data suggest that the modification of functions in plants seems to be related to the external concentration either of undissociated molecules or of anions. Deviations from these relationships might be the result of two or more effects masking or interfering with each other.

How these extremes may be reconciled in the same cells is difficult to understand. One possible explanation is that ions have free entry into the cytoplasm (2). Thus, effects related to the concentration of undissociated molecules would occur in the lipid phase, whereas effects related to the concentration of anions would occur in the aqueous phase.

Another point of view would subscribe to the more usual contention that the cytoplasm is permeable only

to the molecules and that the responses related closely to the concentration of undissociated molecules in the external solution would depend either on undissociated molecules or on anions within the cells. This interpretation leaves the relationship of responses closely related to the external anion concentration dependent on reactions occurring principally at the surface of the protoplast (16).

Although correlations between response and external concentrations of undissociated molecules or of anions are possible, it does not necessarily follow that these relationships reflect activity of the particles within the cells. For example, although the concentration of undissociated acid at various external pH levels remained fairly constant for 50 % inhibition of photosynthesis in response to either 2,4-D or acetic acid at various pH levels, this is not considered evidence that undissociated molecules participated in a reaction within the cells. It could very well have been only the anions within the cytoplasm which brought about the effect. The anions would vary in much the same order as the undissociated molecules, barring changes in the pH of the cytoplasm. The undissociated molecules could have served merely as a form which readily penetrated the cytoplasm.

Assuming that the anion within the cell is the active form or starting point in a series of reactions leading to the inhibition of photosynthesis, another comparison can be made between 2,4-D and acetic acid. Using the concentrations of undissociated 2,4-D and acetic acids determined to be required for 50 % inhibition of photosynthesis at pH 4.0, the anion concentrations in the cytoplasm, which was assumed to be at pH 6.8 (17), were calculated to be 2.35×10^{-2} M and 8.63×10^{-2} M, respectively. This 3- to 4-fold difference is negligible in comparison with the more than 200-fold difference represented by the undissociated molecules. Since there is no reason for believing that the inhibition of photosynthesis in Chlorella is preferentially affected by 2,4-D either as a specific substance or as a plant growth regulator, the nearly complete elimination of difference in activity between the two acids, based on a comparison of anions, is not perplexing.

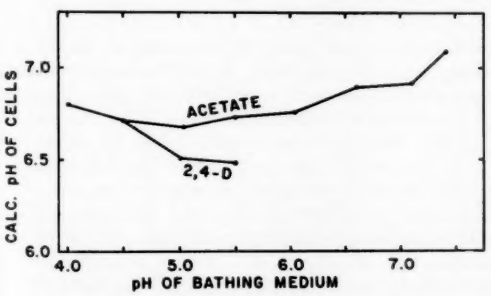

FIG. 4. Variation in pH of cytoplasm of *Chlorella pyrenoidosa* required to maintain a constant anion concentration in the cytoplasm at 50 % inhibition of photosynthesis.

In interpreting the slight deviation of the concentration of undissociated molecules in the external medium from a constant amount for a standard response at several pH levels, there is, in addition to the possibility of a masking or interference previously mentioned, the influence of a change in pH of the cytoplasm. Figure 4 shows the magnitude of change in pH of the cytoplasm required to produce a constant anion concentration in the cytoplasm at the various external pH levels and concentrations of undissociated acid. It may be seen that the maximum change required in tests with acetic acid (0.6 pH unit) would be somewhat higher than that required in tests with 2,4-D (0.3 pH unit). Whether or not such changes in pH in the cytoplasm would be likely is not known.

SUMMARY

The concentration of undissociated 2,4-dichlorophenoxyacetic acid in the bathing medium which produced 50 % inhibition of photosynthesis in *Chlorella pyrenoidosa* increased slightly over the range from pH 4.0 to pH 5.5. Similar determinations for acetic acid showed an increasing amount of undissociated acid required between pH 3.97 and pH 5.0 and a decreasing amount between pH 5.0 and pH 7.42.

The 2,4-D was more than 200 times as effective as acetic acid in inhibiting photosynthesis based on the concentration of undissociated molecules in the bathing medium at pH 4. A comparison of the postulated concentrations of anions in the cytoplasm indicated that 2,4-D was only 3 to 4 times as active as acetic acid in inhibiting photosynthesis.

The amount of acetate anion increasing oxygen uptake by 20 % decreased slightly over the range from pH 4.0 to pH 7.55.

Inhibition of photosynthesis by both 2,4-D and acetic acid appeared to be closely related to the concentration of undissociated molecules in the bathing medium, whereas the stimulation of oxygen uptake by acetic acid appeared to be related to the concentration of the acetate anion in the bathing medium.

Evidence was not available to indicate whether or not the undissociated molecules participated in a reaction within the cells. The role of the undissociated molecule may have been only that of a penetrant.

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CHANGES IN THE ACTIVITIES OF SEVERAL ENZYMES DURING GERMINATION AND SEEDLING DEVELOPMENT IN CORN (ZEA MAYS L.) [1,2,3]

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The literature contains numerous references (e.g., Bonner (1), Van Fleet (18), Weier and Stocking (19)) to the occurrence of catalase, cytochrome oxidase, peroxidase, phosphatase, and polyphenolase activities in higher plants, and an occasional report has appeared dealing with certain of these activities in corn. Thus there have been reports of cytochrome oxidase (4, 9), peroxidase (2), catalase (2, 3, 13), and phosphatase (8) activities in preparations of various corn tissues. In most cases, however, only one stage of development has been considered. The experiments reported here were undertaken when, in connection with other studies in progress at this laboratory, it became necessary to obtain estimates of the five types of activity in preparations of corn embryos, etiolated shoots, and green seedlings. The aim of these experiments was threefold: to adapt published assay methods for use with preparations of corn tissue, to estimate the activities of preparations from corn at the various stages of development listed above, and to study the fractionation of activities effected by high-speed centrifugation.

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Materials and Methods

Seeds of corn (*Zea mays* L.) of the variety, Krug, and the hybrid, L 289 × I 105, were used in these studies. Seeds were soaked for 5 minutes in 1% sodium hypochlorite solution, thoroughly rinsed with distilled water, and for embryo and shoot material were incubated between wet paper towels in Petri dishes in a dark chamber at 24° C. Seeds used for embryo preparations were incubated for a period of 10 hours, which was found to be sufficient to permit easy removal of the pericarp and excision of the embryo. For the production of etiolated shoots, seeds were incubated for longer periods as indicated in table I. Etiolated shoots were excised at the scutellar node, and thus each shoot consisted of the first internode of the stem, the coleoptile, and the unexpanded foliage leaves and shoot apex within the coleoptile. Since an illuminated growth chamber was not readily available, green seedling material was grown near a window in the laboratory. The seedlings were grown in vermiculite and were watered with tap water. For enzyme preparations, seedlings were excised at the surface of the vermiculite, and the remains of the coleoptiles which clung to the stems were removed and discarded. The term green seedling, as used in this report, therefore refers to only that portion of the seedling which is above the coleoptilar node.

The following procedure was used in making the enzyme preparations. Excised embryos, etiolated shoots, and green seedlings were washed once in cold distilled water and were then thoroughly ground in cold mortars with approximately 5 parts of cold water and 0.7 parts of reagent-grade sea sand. The resulting slurries were diluted with cold water to 10 ml per gm of tissue for the shoot and seedling preparations and 20 ml per gm of tissue for the embryo preparations, and were centrifuged for 2 minutes in a clinical centrifuge. Supernatants from this centrifugation were used as the enzyme preparations. The pH of these supernatants was approximately 7.0 for embryo preparations and 5.8 for preparations of etiolated shoots and green seedlings. The supernatants were used without further dilution for the estimation of catalase, phosphatase, and polyphenolase activities, and were diluted 1:5 and 1:10 for assay of cytochrome oxidase and peroxidase activities, respectively. For these dilutions pH 7.0 buffer was employed which was 0.05 M with respect to potassium phosphate, 4×10^{-4} M with respect to $AlCl_3$, and 0.41 M with respect to D-mannitol. Preparations were kept in ice baths as much as possible until enzyme runs were completed. Under the conditions used the preparations exhibited generally excellent stability over the period of 4 to 6 hours required for a series of determinations.

In preliminary work on methods of assay for the various enzyme activities, preparations of etiolated shoots were used and conditions, particularly pH and concentrations of substrate and preparation, were varied over wide ranges. The methods which appeared most satisfactory are described below. It was found that when these assay methods were employed, the relationship between reaction rate and preparation concentration approached linearity over a several-fold range in concentration of preparation.

Catalase activity was estimated by a modification of the titrimetric method presented by Sumner and Somers (16). To 7.4 ml of 0.01 M potassium phosphate, pH 7.0, 0.3 ml of 0.21 M H_2O_2 was added, and the solution was allowed to equilibrate in a bath of ice and water (approximately 2° C). Enzyme preparation (0.3 ml) was added at zero time and 2-ml sam-

Table I

Specific Activities of Five Enzymes in Preparations of Embryos, Etiolated Shoots, and Green Seedlings of L 289 × I 205 Corn

Plant part	Age in days	Ave length or ht	Ave fresh wt/part	Protein N/ml original supernatant	Specific activity *				
					Catalase	Cytochrome oxidase	Peroxidase	Phosphatase	Polyphenolase
		mm	*gm*	*mg*					
Embryo	..	...	0.020	0.690	42	0.39	2.0	0.87	0.03
Etiolated shoot	3	4	0.007	0.720	39	0.74	12.2	0.82	0.11
	4	16	0.040	0.380	36	1.09	18.8	1.31	0.31
	5	31	0.080	0.295	38	1.17	27.7	1.66	0.53
	7	76	0.245	0.165	55	1.32	48.8	2.64	0.85
	9	130	0.520	0.105	87	1.35	69.1	3.82	1.43
Green seedling	8	119	0.60	0.220	7	0.44	22.6	1.43	3.21
	11	220	1.06	0.135	15	0.50	36.3	1.91	3.14
	14	280	1.70	0.100	14	0.50	52.6	2.16	4.60

* Specific activities are expressed in the following units per minute per mg protein N:
catalase—micromoles H_2O_2 destroyed
cytochrome oxidase—micromoles cytochrome c oxidized
peroxidase—increase in optical density at 460 mμ
phosphatase—micromoles *p*-nitrophenol liberated
polyphenolase—increase in optical density at 410 mμ

ples were pipetted into 5-ml portions of 2 % H_2SO_4 at ¼, 3¼, and 6¼ minutes. Samples were titrated immediately to the first faint pink with 0.003 M $KMnO_4$ in 2 % H_2SO_4. Although the pink color faded rather rapidly when samples of the reaction mixture were titrated, acceptable reproducibility was attained by titrating to the end point indicated.

Assay of cytochrome oxidase activity was based on the spectrophotometric method of Hogeboom and Schneider (5), the principal change from the published method being a decrease in the concentration of cytochrome c to 1.33×10^{-5} M. Using this lower concentration permitted the use of water in the blank cell of the Beckman DU spectrophotometer, thus making possible measurements at a narrow slit width (0.02 mm). Increasing the concentration of cytochrome c above 1.33×10^{-5} failed to increase the reaction rate under the conditions of these experiments. Cyanide at a concentration of 10^{-3} M effected complete inhibition of activity. In the assay, 2.57 ml of buffer (0.05 M potassium phosphate, 4×10^{-4} M $AlCl_3$, pH 7.0) and 0.4 ml of reduced cytochrome c solution (10^{-4} M), previously brought to 30° C in a constant temperature bath, were mixed in the Beckman cell, 0.03 ml of enzyme preparation was added at zero time, and optical density readings at 550 mμ were recorded at 1-minute intervals for 6 minutes. The cytochrome c used was purchased from the Sigma Chemical Company.

Peroxidase activity was assayed by a method based on the techniques used by Ponting and Joslyn (12) and Morris, Weast, and Lineweaver (11), with the Beckman spectrophotometer cell serving as the reaction vessel. Buffer (0.01 M sodium acetate, pH 5.6) and guaiacol (0.18 M) solutions were brought to 30° C. A 0.5-ml portion of guaiacol was then added to 2.75 ml of buffer in the Beckman cell, 0.2 ml of cold 0.029 M H_2O_2 was added, and at zero time 0.05 ml of enzyme preparation was added. Optical density readings at 460 mμ were recorded at 1-minute intervals over a period of 5 minutes.

Phosphatase activity was assayed by a modification of the method of Sommer (14). To 5.8 ml of buffer (0.05 M citric acid, 0.075 M ethylene diamine, pH 4.9), 1.0 ml of 0.01 M disodium *p*-nitrophenyl phosphate (Sigma Chemical Company) was added, and the solution was brought to 30° C. At zero time 0.2 ml of enzyme preparation was added, and at 1, 5, and 9-minutes, 1-ml aliquots were pipetted from the reaction mixture into 3-ml portions of 0.1 *N* NaOH. Samples were usually allowed to stand approximately 1 hour after addition to the base, and optical densities at 400 mμ were then read using the Beckman DU spectrophotometer. The extinction coefficient of *p*-nitrophenol in dilute base at 400 mμ was found to be 1.60×10^7 cm² per mole. This value was used in calculating specific activities. The addition of Mg^{++} to a final concentration of 10^{-3} M increased the phosphatase activity of corn shoot preparations only slightly (< 5 %). It appears, therefore, that the principal corn shoot phosphatase can be included with the class II phosphomonoesterases of Sumner and Somers (16).

Polyphenolase activity was estimated by a method patterned after that of Ponting and Joslyn (12) but adapted for use of the Beckman cell as the reaction vessel. Buffer (0.01 M sodium acetate, pH 5.6) and catechol (0.5 M) solutions were brought to 30° C before they were added to the cell. The reaction mixture consisted of 2.9 ml of buffer, 0.5 ml of catechol, and 0.1 ml of enzyme preparation. The enzyme preparation was added at zero time and optical densities at 410 mμ were recorded at 1-minute intervals for 6 minutes.

In experiments concerned with the centrifugal fractionation of preparations with respect to the five enzyme activities, etiolated shoots were ground in unbuffered 0.41 M mannitol. Following the usual low speed centrifugation, supernatants (pH approximately 5.8) were subjected to centrifugation at approximately 40,000 × g for 20 minutes in a Spinco model L refrigerated ultracentrifuge. The sedimented fraction from this centrifugation was washed by resuspending in 0.41 M mannitol and recentrifuging, and was finally suspended in 0.41 M mannitol and diluted to the original volume.

For the determination of protein nitrogen, a modification of the method of Koch and McMeekin (7) was used. Dilutions of the enzyme preparations were treated with equal volumes of 20 % trichloroacetic acid, the resulting precipitates were centrifuged down, washed once with 10 % trichloroacetic acid, and digested with H_2SO_4 and H_2O_2. Nitrogen content of the digests was then determined colorimetrically through the use of Nessler reagent.

Results and Discussion

The results of typical experiments with embryos, etiolated shoots of various ages, and green seedlings of various ages for corn of the single cross hybrid, L 289 × I 205, are presented in table I. Activities listed are based on duplicate determinations. Comparable determinations have been made with embryos and shoots of the variety, Krug, with essentially similar results.

It is apparent from the data that in comparison with shoot preparations, embryo preparations are approximately equal in catalase activity, somewhat lower in cytochrome oxidase and phosphatase activities, and much lower in peroxidase and polyphenolase activities. Seedling preparations, on the other hand, are appreciably lower than shoot preparations in catalase and cytochrome oxidase activities, approximately equal in peroxidase and phosphatase activities, and somewhat higher in polyphenolase activity. In etiolated shoots it appears that there is an increase in the specific activities of the five enzymes with age. Stafford (15) has noted a similar effect of age of etiolated pea seedlings on the activities of several enzymes, one of which was cytochrome oxidase. In the present work, this increase is most pronounced in peroxidase and polyphenolase activities. Some increase in spe-

TABLE II

DISTRIBUTION OF ENZYME ACTIVITIES FOLLOWING HIGH-SPEED CENTRIFUGATION OF A PREPARATION OF 5-DAY ETIOLATED SHOOTS OF KRUG CORN

ACTIVITY	SPECIFIC ACTIVITY *			% TOTAL ACTIVITY			
	CRUDE PREPARATION	SEDIMENT	SUPERNATANT	CRUDE PREPARATION	SEDIMENT	SUPERNATANT	SUM OF SEDIMENT AND SUPERNATANT
Catalase	44.6	84.0	16.1	100	69	21	90
Cytochrome oxidase	1.09	2.48	0.0	100	83	0	83
Peroxidase	32.5	21.1	37.7	100	24	69	93
Phosphatase	1.79	0.40	2.80	100	8	93	101
Polyphenolase	0.73	1.31	0.35	100	66	28	94
Protein N	....	....	....	100	37	59	96

* See footnote, table I, for units.

cific activity with age is noted in green seedling preparations, but the increase found here is generally less than that noted for the shoot preparations. Of particular interest is the fact that embryo preparations are characterized by extremely low peroxidase and polyphenolase activities, while shoots and seedlings—particularly the older ones—yield preparations which are highly active with respect to these two enzymes. As reported by James (6), polyphenolase activity is also very low if not absent in embryos of barley.

The data on protein nitrogen (table I) reveal a decrease in protein content with age in preparations of etiolated shoots and green seedlings. This fact must of course be considered if one is interested in selecting a stage of development which will yield preparations with a high activity per unit volume. The 3-day shoot preparation, for example, is lowest among the shoot preparations in terms of the specific activity of cytochrome oxidase, but it is highest among these preparations in terms of cytochrome oxidase activity per unit volume. Specific activities of the preparations were calculated on the basis of protein nitrogen rather than tissue weight because it is thought that the former means of expression provides a more reliable indication of the enzyme activity per unit of protoplasm.

When crude preparations suspended in 0.41 M mannitol are subjected to centrifugation at 40,000 × g for 20 minutes, a fractionation of activities is effected as indicated in table II. The data presented are based on an experiment with 5-day etiolated shoots of the variety, Krug. Similar results have been obtained with shoots of Krug of other ages, and with shoots of L 289 × I 205. The data show that the sedimented fraction accounts for the bulk of the catalase, cytochrome oxidase, and polyphenolase activities, suggesting that under the experimental conditions used, these enzymes are for the most part associated with cytoplasmic particles. These results agree generally with the work of McClendon (10) who found that in tobacco leaves, cytochrome oxidase activity is to a large extent associated with cytoplasmic particles, and that catalase and polyphenolase activities are at least partially associated with such particles. As shown in table II, the supernatant fraction accounts for most of the phosphatase and peroxidase activities, indicating that if these two activities are associated with any cellular particles, such particles are not readily sedimented under the conditions used. The distribution of catalase and peroxidase activities indicates that in these determinations catalase accounts for little if any of the peroxidase activity, although Tauber (17) has shown that under some conditions catalase can function as a peroxidase.

SUMMARY

Specific activities of preparations of corn embryos, etiolated shoots (excised at the scutellar node), and green seedlings (excised at the coleoptilar node) with respect to five enzymes have been determined. The preparations may be arranged as follows in order of their specific activities: catalase—seedling < shoot, embryo; cytochrome oxidase—embryo, seedling < shoot; peroxidase—embryo < shoot, seedling; phosphatase—embryo < shoot, seedling; and polyphenolase—embryo < shoot < seedling. Etiolated shoots of increasing age yielded preparations of increasing specific activity with respect to the five enzymes. A similar but less pronounced effect of age was noted in experiments with green seedlings. High speed centrifugation of shoot preparations resulted in the sedimentation of a fraction with which were asociated most of the catalase, cytochrome oxidase, and polyphenolase activities of the original preparation, while the supernatant accounted for most of the peroxidase and phosphatase activities.

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THE ISOLATION OF PLANT CUTICLE WITH PECTIC ENZYMES[1,2]

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The isolation of relatively intact sheets of plant cuticle from leaves of several species has been carried out in connection with a study of the interaction of plant cuticle with various foliar applied chemicals. Previous work by Skoss (5) demonstrated that plant cuticle can be isolated by anaerobic bacterial fermentation of leaves. Wood et al (6) observed that filtrates from culture solutions of *Bacterium aroideae* decomposed various plant tissues and sometimes caused the release of cuticle. Chayen (1), Hohl (2), and others have used pectic enzymes as mild macerating agents in anatomical and cytological studies. The present work was an attempt to develop a simplified cuticle isolation procedure based on the use of commercially available pectic enzymes.

MATERIALS AND METHODS

Disks, 1.8 cm in diameter, were punched with a cork borer from freshly harvested leaves of several species and placed in Griffin beakers. The disks were then covered with approximately 2 ml per disk of buffered pectic enzyme solution containing 100 ppm Merthiolate (Thimerosal, Lilly) as a disinfectant. The disks were weighted to the bottoms of the beakers with heavy wire screen and the enzyme solutions were thoroughly aspirated into the tissues, using intermittent vacuum. The beakers containing the infiltrated disks were then placed on a slow rotary shaker (100 rpm - 2.5 cm radius of rotation) in a cabinet maintained at 35° C. After leaf decomposition, isolated cuticles and vascular tissue were collected by passing the resulting suspension through a 10 mesh wire screen. The isolated cuticle disks and tangled masses of vascular tissue retained by the screen were carefully washed from the screen into a liter of water, gently stirred, and sieved again. Three or four screenings were usually sufficient to remove practically all cellular debris. The leaf vascular tissue was sorted with a tweezers from the washed cuticle disks, and the disks were then stored in distilled water containing 100 ppm Merthiolate. Comparisons between treatments were based on the rates at which isolated cuticles appeared in the decomposition medium.

EXPERIMENTAL RESULTS

Among the several commercial enzyme preparations tested, Enzyme 19 (formerly Enzyme 19AP, Rohm and Haas Co.) and Pectinase (Nutritional Biochemicals Corp.) proved most satisfactory. Pec-

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tinase usually decomposed leaf tissue more rapidly than Enzyme 19 but the latter product carried decomposition somewhat further, eventually decomposing cell walls as well as intercellular substance, and hence was more effective on refractory species. Other products, e.g., the Rohm and Haas Pectinol series (including Pectinol 10M), decomposed leaves of susceptible species but were not as active as the two preparations first mentioned.

Decomposition proceeded most rapidly with both preparations in the pH range 3.5 to 4.5. Acetate buffer at 0.1 M was satisfactory. Concentrations of enzymes from 2 to 3 % (w/v) gave maximum rates of cuticle release. Merthiolate had no inhibiting action on these enzyme preparations at concentrations as high as 10,000 ppm. Gentle rotary agitation speeded decomposition considerably but reciprocal or violent agitation resulted in rolled and shredded cuticles. Decomposition was more rapid at 35° C than at room temperature. After leaf decomposition, the beakers contained a suspension of isolated cells and cuticle disks, and tangled masses of leaf vascular tissue (3). In the case of *Citrus* sp., a rigid vascular skeleton was obtained.

Various species responded quite differently to treatment with pectic enzyme solutions. Some released intact cuticles within 3 to 12 hours after being placed in the solution, e.g., *Convolvulus arvensis, Peperomia* sp., *Philodendron* sp. and *Vinca major*. Others also decomposed rapidly but yielded only flecks of cuticle, e.g., *Lactuca scariola, Phaseolus vulgaris, Rumex crispus* and *Sonchus oleraceus*. The small particles of cuticle were not isolated for further study. Another group, including *Citrus* sp., *Coprosma* sp., *Nicotiana glauca, Prunus armeniaca* and *Prunus persica,* decomposed in 12 hours to 3 days and yielded intact sheets of cuticle. The following decomposed very slowly or not at all: *Camellia* sp., *Canna* sp., *Ceratonia siliqua, Cinnamomum camphora, Ficus elastica, Glycine Max, Gossypium hirsutum, Magnolia grandiflora, Platanus acerifolia, Quercus* sp., *Syringa vulgaris, Vitis* sp. and *Zea mays*. Young leaves generally decomposed faster than those that were more mature.

Isolated *Prunus armeniaca* cuticle disks were examined in greater detail than those of other species since large quantities had been obtained for penetration studies. Cuticles from Citrus and Photinia species were relatively thick, those from *Prunus armeniaca,* Vinca and Coprosma species were intermediate in thickness, and those from Peperomia and Philodendron species were quite thin. Cuticle color ranged from white for that of Peperomia and Philodendron species, to greenish-yelow for that of *Prunus armeniaca* and *Vinca* sp., to green for that of *Citrus* sp. In some preliminary experiments involving anaerobic fermentation of Citrus leaves, it was noted that the isolated cuticles were yellow within the anaerobic culture bottle but upon removal changed to green within only a few minutes. *Prunus armeniaca* cuticles exhibited a golden fluorescence under ultraviolet irradiation. With some species, e.g., *Prunus armeniaca* and *Prunus persica,* the cuticles from the lower leaf epidermis were thinner, more flexible, and had a "frosted" appearance when compared with those from the upper epidermis. With others, e.g., Citrus, Vinca and Coprosma species, it was difficult to distinguish upper from lower cuticles without the use of a compound microscope. The outer or external surface of isolated cuticles was more hydrophobic than the inner surface (which was originally contiguous with the epidermal cell wall). Hence, at an air-water interface, cuticles from certain species showed a pronounced tendency to orient themselves with their inner surfaces contacting the water. On heating, *Prunus armeniaca* cuticles apparently volatilized over a rather wide range of temperature. The gross structure of isolated cuticle was not visibly altered by common organic solvents at room temperature. Cold ether and refluxing xylene dissolved certain components of *Prunus armeniaca* cuticle. The xylene extract yielded a brown waxy material with a marked odor suggestive of benzaldehyde. Refluxing with xylene caused the cuticle disks to shrink to approximately one half of their former diameter. Cuticle disks also shrank to approximately two thirds former diameter when maintained in water at 100° C for two hours.

Under the microscope, the projections of the cuticle into the spaces above the epidermal anticlinal cell walls (cuticular flanges) were quite prominent in most species. The cuticle usually appears quite granular under high power (640×). Insect punctures incurred by the cuticle during early stages of leaf expansion apparently give rise to corky or gummy plugs which are surrounded by the outlines of rosettes of epidermal cells. Isolated *Prunus armeniaca* cuticles, although intact to all outward appearances, actually contained many imperfections or cracks through which liquids could readily pass. Some of these imperfections may be present on the functional leaf and may play an important part in the uptake of foliar applied chemicals.

Prunus armeniaca cuticle stained readily with basic dyes at intermediate or high pH values, and with acidic dyes at low pH values. In sorptive properties it resembled a semi-lipoidal cation exchange membrane (4). The permeability and sorptive properties of isolated plant cuticle will be discussed in a subsequent publication.

Discussion and Conclusions

The decomposition of plant intercellular substance apparently results from the presence of pectin-polygalacturonases present in the enzyme preparations. A solution of yeast pectin-polygalacturonase was as effective as either of the two recommended preparations. Pectin esterase (Rohm and Haas Co.) had no effect either alone or in combination with yeast pectin-polygalacturonase. When acted upon by polygalacturonase enzymes, pectinaceous cell walls swell, lose their affinity for ruthenium red, and then disin-

tegrate. The rather broad pH optima for the commercial preparations may reflect the presence of several active enzymes. It is possible that the structure and some of the properties of cuticle were altered to a certain extent by the enzymes employed.

Microscopic examination of leaf cross-sections made with a freezing microtome and stained with ruthenium red or Sudan IV revealed that cuticle isolation occurred most readily with those leaves in which there was a sharp separation, or a pronounced concentration, of pectic materials between the cuticle and epidermal wall. In general, isolation did not occur readily with species which possessed hard, stiff, or leathery leaves, for in such cases the cuticle usually was not sharply defined but graded into the epidermal and subjacent cell walls. It is possible that species which yielded only very small particles of cuticle during decomposition either had a very thin cuticle which fragmented upon release, or possessed an imbricated cuticle composed of many small platelets cemented together by pectic materials. One would expect such an arrangement to result in ready penetration of polar substances.

Summary

A simple and rapid method for the isolation of large amounts of certain kinds of plant cuticle is described. The method involves the decomposition of leaf segments in solutions of commercially available pectic enzymes under certain conditions of pH, concentration, and temperature.

The author wishes to express his gratitude to Dr. H. B. Currier, Dr. A. S. Crafts, and Dr. L. K. Mann for their counsel and many helpful suggestions during this work. He also wishes to thank A. L. Demain for the yeast pectin-polygalacturonase solution and Dr. D. M. Reynolds for supplying certain of the commercial pectic enzyme preparations.

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BRIEF PAPERS

STUDIES ON THE MINERAL NUTRITION OF CASSAVA (MANIHOT UTILISSIMA POHL) [1]

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In spite of the great economic importance presented by the cassava crops to Brazil and to other subtropical and tropical countries, to our knowledge no work has been done to study the mineral nutrition of this plant. Cassava roots are consumed either as such (after being cooked) or as manihot meal. The starch which is extracted from the roots and exported represents an important source of foreign currency to Brazil.

The present experiment was carried out to study the effects of the macronutrients nitrogen, phosphorus, and potassium on the yields as well on the composition of the roots. Preliminary data were presented elsewhere (5).

Plants were grown in sand culture in pots provided with holes for drainage; each pot received 60 kg of acid washed sand. The following treatments with four replicates were used: $N_0P_1K_1$, $N_1P_0K_1$, $N_1P_1K_0$, $N_1P_1K_1$, $N_2P_1K_1$, $N_1P_2K_1$, and $N_1P_1K_2$. Treatment levels were as follows: $N_1 = 35$ gm of NH_4NO_3; $P_1 = 35$ gm of $Ca(H_2PO_4)_2$; $K_1 = 28$ gm of K_2SO_4. Each pot also received 26 gm of $MgSO_4$ and monthly doses of micronutrients as indicated by (3). All salts used were of analytical grade. To avoid excessive salt concentration the treatments were divided into four applications evenly distributed throughout the life cycle of the plants. On July 15, 1952, each pot was planted with a cassava cutting of the variety Branca de Sta. Catarina. The plants were watered daily; the excess water added was allowed to drain off from the sand cultures into containers below each pot, and returned to the surface of the sand on the next watering. The plants were harvested May 15, 1953.

Chemical analyses were made on root samples taken out of each pot. Standard methods were used: starch—determined after (2); ash—by combustion at 500° C; proteins—according to (4), $CuSO_4$ being employed as a catalyst (the conventional factor 6.25 was used to convert nitrogen to protein).

The statistical analysis was carried out by considering first the treatments in which one element was omitted, and afterwards the treatments with double doses; root and shoot data were analyzed separately; treatment $N_1P_1K_1$ was taken as control; the least significant differences (L.S.D.) were calculated at the 1 % level of probability.

Table I shows that as far as yields of both roots and shoots are concerned, the most striking effect was found when P was omitted, as this treatment caused the poorest yields of the whole experiment. The requirement of this element for the phosphorylation of the starch reserves necessary to carry on the vegetative growth during the early stages of development probably accounts for this high response to P addition. Such a response may also in part reflect the amount of P in the cuttings when they were set out in the sand culture.

TABLE I
EFFECT OF THE TREATMENTS ON GROWTH OF CASSAVA

TREATMENT	AVE FRESH WT IN GM	
	ROOTS	SHOOTS
$N_0P_1K_1$	455.00	277.00
$N_1P_0K_1$	265.00	231.25
$N_1P_1K_0$	940.00	761.25
$N_1P_1K_1$	1,460.00	483.75
L.S.D.	469.43	216.19
$N_2P_1K_1$	1,962.50	1,037.50
$N_1P_2K_1$	1,750.00	835.00
$N_1P_1K_2$	1,775.00	757.00
L.S.D.	411.85	296.01

Leaves of the plants grown in the absence of P treatment showed the red purplish color characteristic of P deficiency. The response to N was as large as that observed from the addition of P in the fertilizer. The effect of K in the root yields was much less marked. In the absence of K treatment, the weight of roots decreased whereas that of the shoots increased. Since there is considerable evidence supporting the role of K in the translocation of carbohydrates (1) it seems reasonable to assume that sugars produced in the leaves were consumed in the building up of more green matter rather than being translocated down to accumulate as starch in the roots. Doubling the doses of any element produced a significant increase in yield, the largest response being due to N. However, the discussion following table II indicates this large response is not justified from an economic point of view.

Table II shows that P and N had a large effect on the starch content of the roots. The chemical data showing the effect of P are in good agreement with the loss in yield found in the minus P treatment. Here again the function of P in the synthesis of starch probably accounts for the observed result. Concerning N, it is interesting to compare table I and table II; by doubling the basic nitrogen rate, the highest root yield of the experiment was obtained. However,

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TABLE II

CHEMICAL ANALYSIS OF THE ROOTS *

TREATMENT	DRY MATTER	STARCH		PROTEIN		ASH	
	%	%	gm	%	gm	%	gm
$N_0P_1K_1$	39.31	32.92	150.75	0.62	2.75	1.23	5.96
$N_1P_0K_1$	32.78	25.68	68.56	1.43	3.81	1.16	3.03
$N_1P_1K_0$	37.49	30.19	284.42	1.40	13.32	0.68	6.44
$N_1P_1K_1$	38.96	31.98	465.23	1.13	16.50	1.00	14.45
$N_2P_1K_1$	32.42	24.74	484.94	1.66	32.57	0.86	16.83
$N_1P_2K_1$	37.96	30.31	530.39	1.22	21.41	1.05	18.33
$N_1P_1K_2$	36.47	28.98	512.47	1.17	20.69	1.04	18.45
L.S.D.**	2.18	2.13	73.38	0.23	6.20	0.29	2.27

* Average results of chemical determinations.
** Calculated at the 1 % level of probability.

this increase in yield did not bring any benefit as far as starch is concerned; actually, treatment $N_2P_1K_1$ caused a severe decrease in the starch content from 32 to 24 % and consequently the total amount of this polysaccharide does not significantly differ from the amount obtained with the $N_1P_1K_1$ treatment. The explanation for this fact is probably as follows: when the N supply was raised, a substantial amount of simple carbohydrates were formed and, instead of being polymerized to starch, were combined with the excess of amino groups producing more proteinaceous material. Actually, treatment $N_2P_1K_1$ caused an increase of almost 50 % in the nitrogenous material over the amount found in treatment $N_1P_1K_1$.

SUMMARY

This paper relates the results of a sand culture experiment designed to study the physiological basis for the nutrition of cassava (*Manihot utilissima* Pohl). Chemical analyses of the roots were run to determine the effects of the mineral nutrients, N, P, and K on the composition of the roots. The following conclusions were drawn:

1. Phosphorus and N, under the experimental conditions, proved to be the most important nutrients for cassava. Where P was omitted from the fertilizers, the poorest yields were obtained and the reduction in the starch content paralleled the fall in root weight. Treatment $N_1P_1K_1$ produced 32 % of starch whereas $N_1P_0K_1$ gave 25 %.

2. Where K was omitted from the nutrients, the weight of shoots increased while that of roots dropped. This result can be explained on the basis of the well known K requirement for the translocation of carbohydrates.

3. By raising the N level, the weights of shoots and roots increased. However, the starch content of the roots fell from 32 to 24 % and the increase in root yield did not compensate for such a drop. On the other hand there was a corresponding increase of about 50 % in the protein content where the plants were given the higher doses of nitrogen.

4. Under Brazilian conditions, it appears that perhaps field responses of cassava to P fertilization are due not wholly to low soil P but also to an unusually high requirement of cassava for P.

The authors are indebted to Mr. A. B. Carlton, University of California, Berkeley, for his helpful reading of the manuscript.

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A RAPID, POSTILLUMINATION DECELERATION OF RESPIRATION IN GREEN LEAVES [1]

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Rabinowitch (4) described the possible existence of photostimulation of respiration in photosynthesizing tissue as "a nightmare oppressing all who are concerned with the exact measurement of photosynthesis, and various attempts have been made to bring it to light." In recent years the problem has been pursued vigorously and has become somewhat controversial. Brown (1) and Brown and Webster (2) have reviewed the subject.

If respiration of a green leaf is unaltered during photosynthesis the carbon dioxide evolution rate should be the same immediately following darkening as it is several minutes later. The work reported here shows that in certain species it was initially very rapid but decelerated quickly.

[1] Received July 1, 1954.
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Respiration was measured by monitoring continuously the CO_2 concentration of a fixed volume of air surrounding a single leaf. The apparatus consisted of a leaf chamber, an air pump and an infra-red gas analyzer (Liston-Becker, Model 15, coupled with an Esterline-Angus recording milliammeter) connected in closed series with plastic tubing and ground glass joints. The leaf chamber consisted of the top of a 15 cm Petri dish sealed to a heavy brass plate with modeling clay. The dish had notches cut in the rim to accommodate the petiole and a thermometer. The apparatus was the same as previously described (3) except that the leaf chamber was substituted for the shoot chamber.

A single leaf, still attached to the potted plant, was sealed in the chamber and was illuminated from above by a battery of internal reflector incandescent lamps focused on the chamber through 5 cm of cold running water. Different light intensities were obtained by interchanging screens of white paper. Air temperature within the chamber was maintained between 24° and 26° C by manual control of the flow of cold water through copper tubing soldered to the bottom of the brass base plate.

The routine procedure consisted of allowing the illuminated leaf to reduce the CO_2 concentration as far as it could, that is, until the compensation point was reached. The leaf chamber was then darkened and the subsequent changes in CO_2 concentration were recorded.

The sensitivity of the technique was such that the changes in the CO_2 exchange rate associated with the interruption of a dark period by 1 second of illumination, or with the interruption of illumination by 1 second of darkness, could be detected with certainty. The slight swervings of the tracings are too minute to be reproduced here but are clear and consistent on the recorder charts.

Changes of CO_2 concentration indicated on the recorder lagged approximately 7 seconds behind actual changes in the chamber. The lag was measured by introducing minute quantities of pure CO_2 into the leaf chamber with a microburette and noting the time required for the system to indicate the new concentration.

Leaf temperatures were measured with a copper-constantan thermocouple and potentiometer. The thermocouple leads were threaded into the leaf according to the suggestion of Waggoner and Shaw (5). The thermocouple was held against the lower site of the leaf by the spring tension of the leads.

The initially rapid evolution of CO_2 immediately following darkening of the photosynthesizing leaf and its rapid deceleration are shown clearly by tracing 1 of figure 1. This tracing was made with a leaf of a hybrid tobacco (*Nicotiana sanderae* Hort. × *N. langsdorffii* Weinm.) following illumination at 2,800 fc of white light. It is practically identical with all such tracings (ca 100) made under the same conditions with the same plant and with other plants of the same seed lot. Very similar results were observed

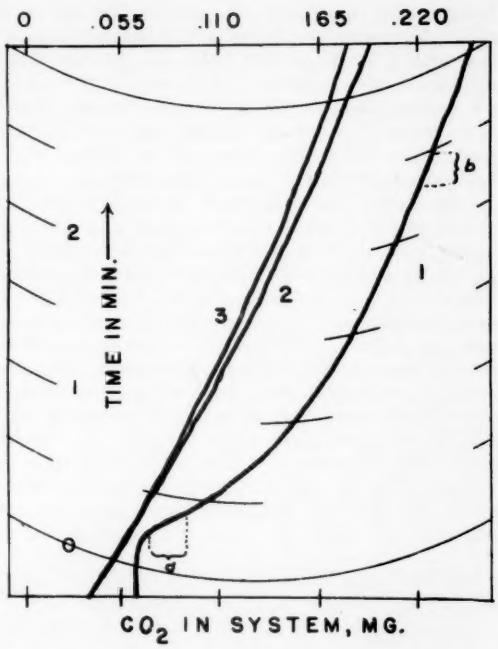

FIG. 1. Composite tracing from milliammeter chart showing CO_2 evolution from a leaf of hybrid tobacco. Curve 1—immediately following illumination (lights off at time 0). Curve 2—in continuous darkness at 30° C. Curve 3—in continuous darkness at 26° C.

consistently with two other tobaccos, *N. langsdorfii* and *N. sanderae*. The following other species have given similar results but with less deceleration evident: pea bean (*Phaseolus vulgaris* L. var. Cornell 716), castor bean (*Ricinus communis* L.), tulip poplar (*Liriodendron tulipifera* L.), white ash (*Fraxinus americana* L.). With chestnut oak (*Quercus muhlenbergii* Engelm.) the effect was slight and inconsistent.

The writer is aware of no other study which has revealed the effect. It seemingly appeared with barley in a single experiment by Weigl, Warrington, and Calvin (6). However, it did not appear in their other experiments, and their results are inconclusive as to the presence or absence of the effect in this species.

To determine whether the phenomenon could be explained in terms of the direct effect of leaf temperature on respiration rate, two studies were made. In the first, respiration rates were measured in continuous darkness with the leaf at 30° and at 26° C (the average light and dark leaf temperatures in this study). Representative results are shown in figure 1. Temperature change, as indicated by a thermocouple in contact with the lower epidermis, was clearly insufficient to account for the changes in respiration rate. There remained the possibility, however, that the internal, respiring tissue was heated more than the thermocouple indicated directly and that the typical respiratory deceleration pattern resulted from in-

ternal cooling of the leaf. If such an effect existed it should have been greatly reduced following illumination with 2,600 fc of blue light, for the blue filter (10 mm of 20 % aqueous $CuSO_4$) reduced the external heating effect of the light markedly and should have reduced the internal heating even more. Average external leaf temperatures were as follows: white light—30.1°, blue light—27.5°, dark—26.5° C.

The initial and subsequent rates of respiration (based on the slopes of segments a and b as shown in figure 1) of a leaf of hybrid tobacco were measured for 20 consecutive cycles of light and dark during which white light at 2,800 fc and blue light at 2,600 fc were alternated. The mean a rates (in mg CO_2/min) were as follows: white—0.1437 ± 0.026 (standard error), blue—0.1348 ± 0.026. The slight difference between mean rates could have occurred by chance alone oftener than once in twenty trials and is considered fortuitous. The mean b rates were: 0.0345 and 0.0339, respectively. The ratios of mean a to mean b were: 4.7 and 3.98, respectively. These results show that the respiratory deceleration pattern could not have been the result of internal cooling of the leaf. The experiment was repeated through 20 cycles with each of two other plants from the same seed lot and planting with almost identical results.

Subsequent experiments have shown that the CO_2 evolution decelerated for approximately 6 to 9 minutes following darkening and that the final stable dark rate was about 75 % of that shown for segment b. Thus the mean initial rate was approximately 5.4 times greater than the basal dark rate.

Obviously more work is needed before an explanation will be found which will demonstrate the compatibility of these findings and those of Brown (1).

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EFFECT OF BORON DEFICIENCY ON NICOTINE FORMATION IN TOBACCO[1]

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Connecticut Broadleaf tobacco plants have been grown in ten liter water cultures deficient in micronutrients for many years (5). A preliminary study of the effects of micronutrient deficiency on nicotine formation was included among the biochemical determinations made with these plants. The micronutrient deficiencies checked were iron, zinc, copper, manganese, boron and molybdenum.

Single plants for assay were selected at random from each group of triplicates. Usually the triplicates were quite uniform visually in every respect. However, only plants showing marked and characteristic symptoms of deficiency were used for analysis. Five series of tests in all were made over a period of more than a year.

A different procedure was followed in sampling in the first trial than in subsequent tests. The method used in this initial experiment was selection of a portion from the middle of the stalk, several leaves midway up the stalk, and the entire root of the plant for analysis. These were dried in a current of air at 80° C for several hours, ground and bottled separately. Each sample was assayed for total alkaloid by Dawson's spectrophotometric method (1, 2). Total alkaloid is equivalent to better than 90 % nicotine in the tobacco variety employed. The results of the several analyses for each plant have been combined and are expressed as percentage and milligrams of total alkaloid for the dry plant as a whole. All other samplings included all the leaves, the entire stalk and the whole root of the plant being assayed.

The first series of determinations revealed significant differences in alkaloid content only with manganese and boron deficiencies. Results on leaves of plants deficient in iron, zinc, copper and molybdenum varied little from those with the control. Examination of table I will show, however, that the minus boron plant displayed a four-fold increase in alkaloid concentration expressed as percent of total dry weight and an increase in quantity of nicotine of about 60 %. This took place despite a 60 % loss in dry-weight yield of the boron-deficient plant. Decreased alkaloid in the minus manganese plant seemed to be a direct consequence of decreased yield, which was equivalent to but 20 % of the control.

As can be seen by comparison with the last group

[1] Received August 13, 1954.

TABLE I

COMPUTED ALKALOID FORMATION IN THE DRIED PLANT AS A WHOLE WITH BORON AND MANGANESE DEFICIENCY OF TOBACCO

Deficiency	Four weeks deficiency			Seven weeks deficiency		
	Total yield *	Total alkaloid		Total yield *	Total alkaloid	
	Gm	%	Mg	Gm	%	Mg
		Spring 1951				
None	...	...	...	144.9	0.13	192.6
B	...	...	...	57.5	0.50	285.4
Mn	...	...	...	27.5	0.08	11.2
		Winter 1951				
None	9.8	0.13	12.6	45.6	0.09	41.9
B	10.8 **	0.13	13.9	43.8	0.36	156.9
Mn	6.6	0.17	10.9	6.0	0.17	10.4
		Spring 1952				
None	14.7	0.14	20.6	99.2	0.12	113.9
B	18.8 †	0.17	31.0	58.9	0.44	259.7
Mn	5.3	0.20	10.6	10.7	0.15	15.9

* Dried in current of air at 80° C for 3 hrs.
** Bud blackening occurred the previous night (early symptom stage).
† No bud blackening at time of sampling (very early symptom stage).

of plants, the method of sampling employed in the first experiment led to results in no way essentially different from those where the whole of the plant parts were included in the sample. Percentages of total alkaloid on a dry weight basis at the early flowering stage of the controls in spring of 1951 were 0.13, 0.50 and 0.08 % with partial plant samples for control, minus boron and minus manganese. In spring of 1952 with whole plant samples, the corresponding values were 0.12, 0.44 and 0.15 %. Agreement was satisfactory in view of the difficulty usually experienced in close control of deficiency levels with micronutrients.

Subsequent tests were divided into early and late samplings. The object was to determine the relation, if any, that increased nicotine synthesis might have to stage in development of minus boron symptoms. Visual boron deficiency symptoms in tobacco have been described by McMurtrey (3). In these experiments they began with excessive branching of the fibrous roots (fig 1) and inhibition of stalk growth, followed very quickly by formation of asymmetric apical leaves. Shortly thereafter basal blackening and death of the apical bud took place. Axillary buds also blackened and died subsequently. Van Schreven (6) has described the internal symptoms of boron deficiency in tobacco. Similar, even though not identical symptoms of boron deficiency, have been studied microscopically and described by Warington for other crops (7, 8). The phenomenon of excessive root branching was noted by each of these investigators. Van Schreven moreover found that the first symptom of boron deficiency in tobacco occurred in the root. Figure 1 illustrates the difference in appearance of the fibrous roots detached at the point of emergence from a tap root in control and minus boron plants. Solt and Dawson (4) found that nicotine formation was confined largely to the apices of shoots and roots. Though boron deficiency in tobacco resulted in death of almost all apices in the shoots, the concomitant large increase in number of root tips resulted in a large net increase in growing points for the plant as a whole.

The first early samplings were made with plants grown in winter. The poorer light at this season of the year was, as usual, reflected particularly in decreased growth of controls. It can be seen also that no rise in alkaloid was evident at the bud blackening stage of the minus boron plant when grown in winter. Three weeks later, however, the picture was almost identical with the first series, except for a minor difference caused by the much poorer growth of the control.

Another repetition of these tests in the spring of 1952 again gave similar results. The earlier samples were taken just prior to the necrotic or bud blacken-

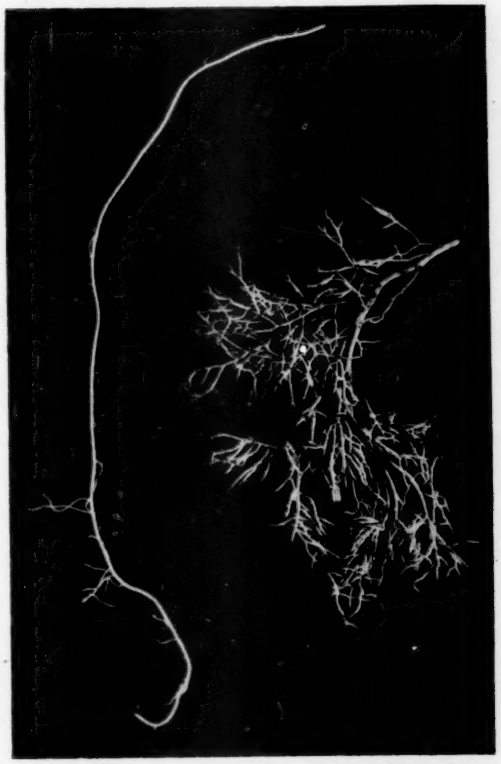

FIG. 1. Type and extent of branching in fibrous roots detached at point of emergence from tap roots. The root on the left is from a control plant and the root on the right from a minus boron plant. Flowering had just begun in the control plant at the time the root was detached.

ing stage of boron deficiency. Excessive root branching and leaf asymmetry symptoms were present but had not reached their maxima. Nicotine content in the minus boron plant had increased about 50 % in quantity on a whole plant basis even at this very early stage before development of symptoms of necrosis. Three weeks later the sampled plants again showed the same relationships found during the two previous series of tests.

The ratio of root to total plant dry-weight varied consistently with type of deficiency in these experiments. At time of flowering it ranged from 8.9 to 11.8 % in the controls; from 11.4 to 17.0 % in minus boron; and from 3.7 to 7.3 % in minus manganese plants. The average values at this stage of growth were 10.7, 14.1 and 5.9 %, respectively, for these three types of plants.

It would appear, therefore, that these data correlate well with the findings of Solt and Dawson (4) that nicotine formation is associated primarily with apices of shoots and roots. Nicotine began to accumulate rapidly shortly after excessive root branching had begun (prior to bud necrosis) in the minus boron plant, and increased rapidly with further aggravation in symptoms. The final result was a rapid and parallel increase in number of root-tips and quantity of nicotine produced that was strikingly greater than for either control or minus manganese plants.

The determinations of total alkaloid discussed herein were made by Dr. R. F. Dawson. The writer wishes to express his appreciation of Dr. Dawson's kindness in both this respect as well as for aid given in preparation of this manuscript.

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THE ROLE OF 2,4-DICHLOROPHENOL IN THE DESTRUCTION OF INDOLEACETIC ACID BY PEROXIDASE[1]

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An enzyme system capable of destroying indoleacetic acid (IAA) has been reported to occur in some plants (4, 10, 12). Further reports (1) have shown that this activity may be reproduced by a crude or crystalline preparation of peroxidase from horseradish, together with added H_2O_2; evidence is presented that the indoleacetic acid oxidase of pea seedlings consists of a peroxidase with a physiological source of peroxide.

The present paper will report results which indicate that a cofactor, mediating the destruction of IAA by peroxidase, is a requirement for activity of certain horseradish preparations. This unknown cofactor may be effectively replaced by 2,4-dichlorophenol (DCP). It will also be shown that an effective peroxidase from Xanthium leaves is incapable of destroying IAA even in the presence of DCP, indicating a difference in the nature of the peroxidase obtained from horseradish and Xanthium.

An extract containing active peroxidase was prepared from dehydrated horseradish obtained from a local food market. The dehydrated powder was ground with a mortar and pestle in water (20 mg/ml) and the particles centrifuged off. The resultant aqueous extract was tested for peroxidase activity by the method of Siegel and Weintraub (8). The enzyme preparation and peroxide were added to potassium phosphate buffer (pH 6.0; final conc. 0.05 M) in a colorimeter tube and the transmission set at 100 %, using a 420 $m\mu$ Evelyn filter. At 0-time, 0.2 ml of freshly prepared 5 % pyrogallol was added to a final volume of 10.0 ml and the rate of color development measured. Control tubes, lacking H_2O_2, indicated a very slow autoxidation and no oxidase enzymes active on pyrogallol. The rate of enzymatic oxidation of pyrogallol is initially linear, but falls off as the purpurogallin accumulates. The horseradish prepared as above was found to have a high peroxidase activity (table I).

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TABLE I

PEROXIDASE ACTIVITY OF CRUDE XANTHIUM EXTRACTS AND HORSERADISH EXTRACT *

XANTHIUM EXTRACT (FRESH WT)	HORSERADISH EXTRACT (DRY WT)	H_2O_2 (0.03 %)	OXIDATION OF PYROGALLOL (TO 50 % TRANSMISSION)
mg	*mg*	*ml*	*sec*
36	..	0.3	33.4
18	..	0.3	59.0
9	..	0.3	98.1
18	..	..	∞
..	2	0.3	70.2

* Total volume of each reaction mixture was 10 ml.

To compare this system with that reported by Galston et al (1), the horseradish peroxidase was incubated with IAA in the presence of peroxide, and the amount of IAA remaining was measured at various time intervals. The concentration of IAA was measured by a modification of the Salkowski test (3). One ml aliquots of the IAA solution were added to 5.0 ml of a solution of 0.05 M $FeCl_3$ in 20 % $HClO_4$. The red color was allowed to develop 20 minutes in darkness (bright light seemed to interfere with color development) and the absorption was measured in an Evelyn colorimeter, using a 540 $m\mu$ filter. No destruction of IAA could be detected in this system (table II).

The concentration of the enzyme extract was kept low in the IAA incubation mixture (equivalent of 20 mg extract/reaction flask) to prevent interference with light transmission in the colorimeter. An aliquot of the incubation mixture was removed at 0-time and measured to get the initial reading. The initial assay was not significantly affected by the presence of either plant extract or the peroxide. It was found necessary to determine the maximum amount of H_2O_2 which may be added without interfering with the availability of IAA (9).

On the basis of the work of Goldacre, Galston, and Weintraub (2), DCP was added to the IAA incubation mixture. On addition of DCP (10^{-5} M) it was found that the peroxidase system would destroy IAA at a rate comparable to that previously reported (table II). Mn^{++} (10^{-3} to 10^{-5} M) had no effect on the rate of destruction of IAA (12). The effective horseradish system required H_2O_2 for activity and could be inactivated by boiling the horseradish extract.

Crude aqueous extracts of young leaves of *Xanthium pensylvanicum* were found to have a high peroxidase activity (table I), as measured by the oxidation of pyrogallol. However, they were found to be completely inactive on IAA. In order to test for the presence of a possible inhibitor in the Xanthium extract, a portion of the extract was added to an active horseradish system (table II). The addition of Xanthium extract resulted in the complete inhibition of the destruction of IAA. Since this Xanthium extract was itself effective in the oxidation of pyrogallol, it could not be expected to interfere with the peroxidase activity of the horseradish enzyme, that is, the inhibition could not be directly on the enzyme activity.

The Xanthium extract was then centrifuged for one hour at approximately 20,000 × g, fractionated with ammonium sulfate (retaining the fraction precipitated between $\frac{1}{3}$ and $\frac{2}{3}$ saturated) and dialyzed against water. This partially purified, active peroxidase no longer inhibited the horseradish peroxidase destruction of IAA. A Xanthium peroxidase system was then prepared, using the partially purified enzyme and DCP (10^{-5} M), and to this was added $MnCl_2$ and boiled horseradish enzyme in all combinations. In no case could the destruction of IAA be detected.

It is concluded that the effective peroxidase from horseradish differs from the peroxidase prepared from the Xanthium leaves, since the enzyme from Xanthium could not replace that from horseradish in the destruction of IAA. The occurrence of more than one peroxidase enzyme has been reported by Theorell

TABLE II

DESTRUCTION OF IAA BY DEHYDRATED HORSERADISH ENZYME AND THE EFFECT OF XANTHIUM EXTRACTS ON THIS ACTIVITY *

HORSERADISH ENZYME (DRY WT)	DCP (M)	Mn^{++} (M)	CRUDE XANTHIUM EXTRACT (FRESH WT)	PURIFIED XANTHIUM EXTRACT (FRESH WT)	MINUTES				
					0	30	60	100	180
mg			*mg*	*mg*	*IAA in μg/ml*				
10	10^{-5}	10^{-5}	...	...	50	25	17	10	..
10	10^{-5}	..	...	...	50	22	17	12	..
10	..	10^{-5}	...	...	50	50	50	50	..
10	..	..	...	...	50	50	50	50	..
10	10^{-5}	..	...	...	50	..	20	..	14
10	10^{-5}	..	300	...	50	..	50	..	50
10	10^{-5}	..	...	305	50	..	21	..	12
..	10^{-5}	..	...	305	50	..	50	..	50

* The incubation mixture originally contained 50 μg/ml IAA and 0.0003 % H_2O_2 in a total volume of 20 ml.

(11) and several peroxidases of differing activities have been recently reported (5, 7). Since the addition of boiled horseradish extract failed to activate the Xanthium peroxidase system, the only alternative explanation would seem to be that a second thermolabile factor is present in the horseradish preparation but lacking in the Xanthium extract.

Previous work (1) has not shown added DCP to be an absolute requirement for the oxidation of IAA by peroxidase. The enzymes used in the earlier studies were a crude extract from horseradish roots and a commercial crystalline peroxidase preparation. The fact that the horseradish root preparation was effective would indicate that this plant organ contains an adequate amount of an effective cofactor. The observation that the crystalline peroxidase had no cofactor requirement may be explained by the observation of Galston et al (1) that this preparation was far from being a pure enzyme. The cofactor may well have been retained in this preparation, but lost during the commercial preparation of the dehydrated horseradish used here.

An enhancement of the activity of IAA oxidase from pea seedlings by DCP has been reported (2). It was concluded by the authors that DCP and certain of its analogues act either as cofactors for the IAA oxidase or, less likely, as electron carriers in the oxidation system. The present experiments indicate that the DCP is effective in the peroxidase portion of the IAA oxidase, rather than on the physiological generation of peroxide. DCP was found during the present work to have no effect on the activity of peroxidase acting on pyrogallol. It may be concluded then, that a cofactor which may be replaced by DCP, acts as an intermediate in the oxidation of IAA by horseradish peroxidase, possibly analogous to the oxidation of Mn^{++} by peroxidase, via phenols, as reported by Kenten and Mann (6).

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NEWS AND NOTES

THE ILLINOIS AND PURDUE SECTIONS: The Illinois and Purdue Sections of the American Society of Plant Physiologists will hold a joint invitational meeting on the campus of the University of Illinois, June 13 and 14, 1955. It is planned to organize the Midwestern Section of the American Society of Plant Physiologists at this meeting. Invited papers on photosynthesis, auxins, and plant nutrition will be read. Round-table discussions will be held on photoperiodism, fruit set, respiration, herbicides, cell wall physiology, postharvest physiology, water relations, and the physiology of parasitism.

The meeting is open to all interested persons. Write John B. Hanson, Department of Agronomy, University of Illinois, Urbana, Illinois, for registration and housing information.—JOHN B. HANSON, Secretary.

ANNUAL REVIEW OF PLANT PHYSIOLOGY, VOLUME V.—D. I. Arnon, Editor. Annual Reviews, Inc., Stanford, California. 399 pages. 1954. $7.00.

The following fourteen articles are included in the fifth volume of this series: Mechanism of Action of Micronutrient Elements in Enzyme Systems by W. D. McElroy and A. Nason; Nutrition by Foliar Application by D. Boynton; Mineral Nutrition of Phytoplankton by B. H. Ketchum; Soil Conditioners by J. H. Quastel; The Photosynthetic Function of Pigments Other than Chlorophyll by L. R. Blinks; Localization of Enzymes in the Cells of Higher Plants by D. R. Goddard and H. A. Stafford; The Physiology of Plant Tumors by A. C. Braun; Tropisms and Nastic Movements by L. Brauner; Dormancy in Woody Plants by R. M. Samish; Postharvest Physiology of Fruits and Vegetables by W. T. Pentzer and P. H. Heinze; Physiological Aspects of Fungus Diseases of Plants by P. J. Allen; The Role of Plant Physiology in Plant Geography by H. L. Mason and P. R. Stout; Photosynthesis by R. Lumry, J. D. Spikes, and H. Eyring; Occurrence, Formation, and Inactivation of Auxins by S. A. Gordon.

Five of the articles are characterized by a strongly biochemical tone while the other nine cover a wide range of broad horticultural-physiological relationships. At least two reviews, those by Braun and Allen, will have unique appeal for plant pathologists.

In their stimulating and provocative treatment Mason and Stout emphasize the lack of physiological research for the solution of problems of the plant geographer. They submit that this is the result of the plant geographer's lack of physiological training or lack of sufficiently well-established physiological principles, the reluctance of physiologists to carry the laboratory into the field, and the sheer complexity of environmental relations. The authors advocate that more attention be given to fundamental processes in individual plants and finally consider applications of physiological principles to plant geography, pertinent research revealing genetics of habitat relations, and experiments dealing with physiology of floristics.

Photosynthesis, clearly eligible for reconsideration by virtue of the abundance of published research in this area, deals this time with pigment photochemistry, structure and composition of chloroplasts, energy transfer mechanisms, the Hill reaction, and quantum requirements. These aspects of photosynthesis are considered in papers by Blinks and by Lumry et al.

Of general interest are the references to dietary aspects of Chlorella and its adequacy as an amino acid source for animals. Brief attention is directed to pilot plant experimentation on algal culture for food.

Pentzer and Heinze have contributed the first review since 1950 on postharvest physiology of fruits and vegetables. The authors have carefully avoided the vast amount of literature dealing with postharvest changes in composition and have emphasized only the functional diseases related to volatile emanations, volatile emanations and respiration, chilling injury, and the effects of growth substances on postharvest behavior. A feature of this review is the rather complete attention given to the history, character and control of apple scald.

In reading prior volumes it has often seemed that authors could do more by occasionally digressing from a running account to present the distillates of their experience through the introduction of compiled tabular or illustrative material to show trends and significant relationships. The reviewer was therefore delighted to find that McElroy and Nason, Goddard and Stafford, and Gordon have brought much meaningful data from many sources into their condensed and valuable tables. These efforts will be appreciated.

In some papers of volume 5, the pagination is incomplete. The citations might be more valuable if the pages for the beginning and end of a paper were given, but in any case, the lack of consistency between reviews within this volume might be questioned. Furthermore, the addition of the title of the paper would be useful, but since the present policy is common to most or all Annual Reviews, it will continue to be debated by some readers.

Like each of the prior volumes, this one is indeed a significant compilation that will be welcomed by a wide array of plant scientists. Despite the indicated preference for some minor changes in format, the reviewer is deeply indebted to authors and editors for maintaining the continuity of very high standards that were set by the predecessors of this volume.

Volume 5 has excellent subject and author indices and cites over two thousand pertinent papers.—THEODORE T. KOZLOWSKI, *University of Massachusetts, Amherst, Massachusetts.*

PREPARATION OF MANUSCRIPTS FOR PUBLICATION IN PLANT PHYSIOLOGY

The following suggestions are made to authors writing papers for this journal in order to minimize revisions and editorial corrections. Though many points may appear arbitrary or trivial, attention to details is necessary to secure uniformity of format and style. The membership dues and subscription fees go almost entirely to publication costs, as all of our editors give their services without financial recompense. Hence the cooperation of authors in preparation of manuscripts minimizes expense and permits greater liberality as to length and number of papers published. Authors will find the following concise publications useful in preparation of manuscript copy: A. J. Riker, The Preparation of Manuscripts for Phytopathology. PHYTOPATHOLOGY XXXVI, pages 953–977, 1946; and S. F. Trelease, The Scientific Paper: How to Prepare it. Second Edition. Williams and Wilkins Company, Baltimore, Maryland, 1951.

1. Length. The allowable length of papers is 10 printed pages, with tables, figures, formulae and equations not to exceed 25 % of the total. Authors will be billed at cost for overrun and excess of figures and tables.

2. Format. In the preparation of typescripts, authors are requested to examine a January, 1955, copy of the journal of PLANT PHYSIOLOGY to note particularly the position of headings, general organization, punctuation, abbreviations and the methods of citing literature in order to bring the script into conformity with the general style of the journal. Leave a three inch margin at the top of the first page, liberal side margins and double spacing of lines to permit proofmarking and required editorial changes. Wherever possible metric units and centigrade temperatures should be used. If it is essential to use English units, such as in pounds per acre, include a conversion factor to metric units the first time the English units are used.

3. Headings. Leave all headings unscored and scientific names without underlining, as the editors will proofmark these items in accordance with the practice of the journal. Generic names used alone are usually capitalized but not italicized. In general, do not underscore any lines.

4. Footnotes. Avoid footnotes as far as possible by placing such items under acknowledgments or at end of summary. When footnotes are used, they should be numbered with Arabic numerals.

5. Figures. Copy for figures (photographs and drawings) should consist of 2 sets and should not exceed $8\frac{1}{2} \times 11$ inches. Retain original drawings as a safeguard against loss. Author's name and figure number must be written on the back of each figure. Do *not* put legends into or on the figure, either front or back. Type all legends *double* spaced, properly numbered to correspond to the figures, on a *separate sheet* of your manuscript. Graphs and drawings should be inked with fairly heavy lines and with lettering of approximately uniform size to insure clarity of printed figures after reduction. Photographic prints should be submitted on gloss-finish paper. Limit copy to desired visual details, trim off irrelevant foreground and minimize blank space, as charges for cuts are based on area. The editor will proofmark copy to determine actual size of printed figures.

Authors are asked to group figures whenever possible so that two, three, four or six figures can be reproduced by a single zinc etching. Do not mix half-tone and zinc etchings in the same grouping. Authors of manuscripts can save the journal approximately $1,000 a year by properly grouping their figures.

6. Tables. Place each table on a separate page; do not crowd too much material into a single table. Note the size of the printed page, and that tables should be designed to fit into one column, i.e. 3 inches in width, wherever possible, although full page tables, $6\frac{1}{8}$ inches wide can be used where required. In no case are tables to be printed turned 90° on the page. Use horizontal lines (rules) at top and bottom, but omit or employ such rules sparingly in the interior of the tables. Rules and spaces (leads) are usually used in the final printed tables to separate sets of data. For footnotes to tables use the asterisk, double asterisk, dagger, double dagger and section in this sequence as needed. Tabular material should be used sparingly because of its high cost.

7. Acknowledgments. Place acknowledgments at the end of the paper just below summary.

8. Address. The name of your institution and its address should be listed just below the names of the authors.

9. Literature Cited. Use separate pages for the literature cited and type *double spaced*. Give complete citations, author, title, journal name, volume number, inclusive pages and year. (See the literature lists in the January, 1955, issue of the journal for examples.) Punctuate according to the examples, and leave no citations incomplete. Write out names of states in full. Note proper abbreviations of journals. An error frequently noted in manuscripts with long bibliographies is failure to mention in the text all citations listed. Authors are requested to check all citations in the bibliography by careful proofreading of manuscripts prior to submission.

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of the receipt of the revised manuscript and not the original date.

11. Galley Proof. Correct all errors clearly and return galley promptly to editor. Authors will be billed at cost for alterations in the galley proofs, other than corrections of printer's errors, involving excessive changes from manuscript copy.

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